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(54) Title: CONTROL OF *IL4* PRODUCTION AS A THERAPEUTIC REGULATOR OF IMMUNE FUNCTION

(57) Abstract

Therapeutic compositions that include small molecule affectors of CP2 function, particularly inhibitors or activators of CP2 production or of complex formation between CP2 and a CP2 recognition element in the *IL4* promoter; methods of screening for such affectors of CP2 function; and methods of use of such compositions are disclosed. The inhibitors or activators are useful as therapeutic agents in methods of treatment of immunological disorders, to restore appropriate immunological balance. Also disclosed are methods of treatment using gene therapy, such as using CP2 cDNA to transform cells from Th1 to Th2, to decrease a harmful inflammatory response in a patient, or using CP2 dominant negative cDNA to transform cells from Th2 to Th1, in order to induce an inflammatory response.

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CONTROL OF *IL4* PRODUCTION AS A THERAPEUTIC REGULATOR OF IMMUNE FUNCTION

FIELD OF THE INVENTION

This invention relates to regulation of the mammalian inflammatory response and particularly to regulation of cytokine control thereof.

GOVERNMENT RIGHTS

Part of the work leading to this invention was carried out with United States Government support provided under grants from the National Institutes of Health, Grant Nos. RO1 GM49661 and DK-37513. Therefore, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

IL4 is the prototypic member of a family of cytokines able to modulate the differentiation and the biologic activities of cells of the hematopoietic lineage, including T cells (Boulay and Paul, 1992). These cytokines, including *IL3*, *IL5*, *IL13* and granulocyte-macrophage colony-stimulating factor, are coexpressed in the T helper (Th)2 subset of $CD4^+$ T cells and in $Fc\varepsilon RI^+$ cells (*i.e.*, basophils and mast cells) (Paul et al., 1993; Paul and Seder, 1994). By secreting *IL4* and related cytokines, these cells play a major role in the regulation of humoral immunity and in the development of allergic/inflammatory responses (Paul and Seder, 1994). In contrast, Th2 and $Fc\varepsilon RI^+$ cells do not produce IL-2 or interferon (IFN)-γ, which are typically associated with the Th1 subset of T cells and play a critical role in the development of cell-mediated responses (Paul et al., 1993; Paul and Seder, 1994). *IL4* itself, perhaps produced by cells other than T cells, such as basophils, mast cells, or $CD4^+NK1.1^+$ cells, is an essential signal for the preferential expression of a Th2 phenotype, while it interferes with Th1 cell differentiation and function (Paul et al., 1993; Paul and Seder, 1994; Yoshimoto and Paul, 1994).

Although several studies implicate a role for differential signal requirements in the generation of Th1- and Th2-related

cytokines (Fitch et al., 1993), the intracellular pathways leading to lineage-restricted *IL4* expression are still elusive. It is, however, feasible that the commitment towards an *IL4*-producing phenotype is the outcome of a complex array of transcriptional activators and/or repressors rather than the effect of a single protein. Elucidation of the most important activators and/or repressors could lend important insight into feasible mechanisms for influencing that commitment and thus controlling preferential expression of a Th2 phenotype and the consequent inflammatory response.

SUMMARY OF THE INVENTION

We have determined that CP2, a 502-aa nuclear protein that was originally identified as a factor binding to at least two elements within the murine α-globin gene promoter and able to activate transcription from α-globin promoter-driven templates *in vitro* and *in vivo* (Lim et al., 1992), is also, unexpectedly, an integral component of a transcriptional complex forming in Jurkat cells on an *IL4* promoter region previously shown to interact with other factors, including NFAT-1, CBF, and HMG I(Y). Additionally, we have shown that *IL2* transcription is repressed in cells overexpressing CP2. These results suggest that CP2 is an important participant in differential cytokine gene expression in human T cells. Furthermore, we have discovered that an alternatively spliced CP2 variant lacking the DNA-binding domain functions as a dominant negative antagonist of CP2 in regulating IL4 production.

Thus, the invention features therapeutic compositions that include small molecule affectors of CP2 function, particularly inhibitors or activators of CP2 production or of complex formation between CP2 and a CP2 recognition element in the *IL4* promoter, and methods of use of such compositions. The inhibitors or activators are useful as therapeutic agents in methods of treatment of immunological disorders, to restore

appropriate immunological balance. The invention also includes methods of screening for such affectors of CP2 function.

The invention further includes methods of treatment using gene therapy, such as using CP2 cDNA to transform cells from Th1 to Th2, to decrease a harmful inflammatory response in a patient, or using CP2 dominant negative cDNA to transform cells from Th2 to Th1, in order to induce an inflammatory response.

BRIEF DESCRIPTION OF THE FIGURES

Other features and advantages of the invention will be apparent from the following detailed description thereof and from the claims, taken in conjunction with the accompanying drawings in which:

Fig. 1 shows the effect of CP2 overexpression of *IL2* and *IL4* promoter activity in transiently transfected Jurkat cells;

Fig. 2A is a schematic diagram of CP2 primary structure, of the structure of nine COOH-terminal truncations of CP2 and of the structure of a ΔElf-1 deletion of CP2;

Fig. 2B shows the effect of COOH-terminal truncation on CP2 activity. Mean ± SEM fold induction of Δ1 through Δ9 cloned into a pRc/CMV mammalian expression vector and cotransfected in Jurkat cells with a p/L4.311 reporter plasmid from three independent experiments is indicated relative to samples cotransfected with a noncoding Prc/CMV vector (designated by the dashed horizontal line). Full-length CP2 expression vector is designated by FL;

Fig. 2C shows the effect of Elf-1 factor deletion on CP2 activity under the same conditions as Fig. 2B;

Fig. 3A is a schematic representation of the *IL4* promoter and of PCR-generated deletional mutants;

Fig. 3B shows the effect of the *IL4* deletional mutants of Fig. 3A on constitutive and induced CAT expression. Jurkat cells were transiently transfected with 1 μg each of the p*IL4*.741 reporter plasmid or its deletional mutants (p*IL4*.311 through p*IL4*.65) and 2 μg of a Prc/CMV-CP2 expression vector or its corresponding noncoding control (indicated by the dashed line).

Mean ± SEM fold induction in three independent transfections are shown;

Fig. 4A shows the nucleotide sequence of the *IL4* promoter regions required for full (225-176) and partial (175-176) transactivation *in vivo*. Boxed sequences (*Box II*, *ISRE*, *P3*, *CCAAT*$_d$, and *P2*) have been characterized as competent recognition sites for the indicated transcription factors. Also shown are oligonucleotide probes *225-176*, *195-146*, and *175-146*, spanning the *IL4* promoter regions included between the indicated bp;

Fig. 4B is a representation of binding of enriched, bacterially expressed CP2 (rCP2) to a 5′ end-labeled α-globin canonical site and to the *IL4* oligonucleotides shown in Fig. 4A;

Fig. 4C shows $(OP)_2Cu^+$ footprinting of a 195-146 oligonucleotide, 5′ end-labeled on the coding strand;

Fig. 4D shows alignment of the *IL4* promoter region protected by RCP2 with a series of CPRE identified in some cellular and viral promoters. The two CNRG boxes are indicated in bold letters. Shown are the distal and proximal CPRE from the mouse α-globin promoter, high-affinity sites from the rat γ-fibrinogen promoter and the HIV LTR, and a low-affinity site from the MHC class II Ea promoter. Also shown is an element within the mouse *Cyp* 2d-9 (steroid 16α-hydroxylase) gene recently found to bind the CP2-related protein LBP-1a (Sueyoshi et al., 1995). Numbers indicate the positions relative to the transcription initiation sites;

Fig. 5A shows binding of Jurkat nuclear proteins to a 5′ end-labeled α-globin or a 195-146 oligonucleotide;

Fig. 5B shows the effect of RCP2 on the binding of Jurkat nuclear proteins to a 195-146 probe. Jurkat nuclear extracts (5 μg) were incubated with the probe with (lane 2) or without RCP2 (lane 3). The white arrow to the left of the gel indicates the complex formed with RCP2 alone (lane 1), while the black arrow indicates the appearance of an additional complex (shown in lane 2) of intermediate mobility between the NFAT-1/CBF complexes;

Fig. 6A shows detection of endogenous CP2 in complexes forming on full-length PCR-generated *IL4* promoter fragments via Dnase I footprinting of an *IL4*.265 deletional fragment, 5′ end-labeled on the noncoding strand. The positions of protected nucleotides are determined by alignment with a Maxam-Gilbert G+A ladder of the same fragment. Numbers to the left of the gel refer to the nucleotides around which elements characterized so far in the human or mouse promoters (schematized to the far left) are centered. Shown is the pattern of Dnase I cleavage of free DNA (F) and DNA incubated with 20 μg Jurkat nuclear extracts (JKT) or with increasing amounts (10, 30, and 100 ng) of RCP2. A footprint between bp -153 and -177 and a hypersensitive region upstream of bp -180 in samples incubated with RCP2 are outlined to the right by a dashed line and a solid line, respectively;

Fig. 6B shows EMSA of PCR-generated, PCR-labeled *IL4* promoter deletional fragments with Jurkat nuclear extracts. Following exposure to the probes, extracts (2.5 μg) were incubated with equivalent amounts (1 μg protein) of rabbit preimmune serum (PS) or the indicated antibodies. The arrow to the left indicates a CP2 immunoreactive complex selectively forming on an *IL4*.225 probe; and

Fig. 6C shows the results of an experiment in which prior to incubation with an *IL4*.225 probe, 1 μg Jurkat nuclear extracts (JKT; lanes 1-6) or 20 ng RCP2 (lanes 7-12) were exposed to a 50-fold molar excess of a panel of oligonucleotides spanning the indicated regions of the human *IL4* promoter. The arrows to the left indicate complexes whose formation is selectively diminished by competition with a 195-146 oligonucleotide.

DETAILED DESCRIPTION OF THE INVENTION

As will be shown in the experiments described herein, we have determined that CP2 is a critical and relatively specific transactivator of the *IL4* gene in human T cells. Our conclusions are supported by the following lines of evidence: 1) *IL4* promoter activity is markedly enhanced, while *IL2* promoter activity is

repressed, in Jurkat cells overexpressing CP2; 2) overexpression of a CP2 dominant negative, specifically inhibiting CP2 binding and function, represses *IL4* promoter activity; 3) transactivation by CP2 maps to an *IL4* promoter region protected by rCP2 in footprinting experiments; and 4) native CP2 is an integral component of a transcriptional complex forming exclusively on *IL4* promoter fragments including the region required for optimal CP2 binding and function.

Overexpression in human T cell lines/clones has been one means for investigating the role of distinct factors in *IL4* transcription (Casolaro et al., 1995; Davydov et al., 1995; Ho et al., 1996; Luo et al., 1996). Here we show that overexpression of CP2 results in a significant increase of *IL4* promoter-driven reporter gene expression in the human T cell line Jurkat. *IL4* activation by CP2 appears to be selective and not merely due to stimulation of shared biochemical pathways in Jurkat cells, since *IL2* promoter- or HIV LTR-driven transcription in the same cells can be markedly repressed (Zhong et al., 1994). While repression by the ΔElf-1 dominant negative suggests that endogenous CP2 critically accounts for *IL4* constitutive transcription in Jurkat cells, transactivation by CP2 was less marked in Jurkat cells stimulated with A23187 and/or PMA. On the other hand, ΔElf-1 CP2 overexpression also repressed *IL4* transcription in stimulated cells. As previously shown in HeLa, K562 and MEL cells (Lim et al., 1993), CP2 is constitutively expressed in the nuclei of Jurkat cells. Stimulation of these cells did not apparently affect CP2 expression and/or binding, with the only exception being a slight but noticeable downregulation by PMA. Therefore, it appears that CP2 accounts for the formation of a constitutive *IL4*-specific transcriptional complex, as shown in Figure 6B, while inducible factors, possibly interacting with CP2, might mediate *IL4* activation in stimulated cells.

We further show that a low-affinity CP2 recognition element (CPRE) is located between bp -177 and -158 of the human *IL4*

promoter. This region includes a sequence ($^{-174}$CTGATTTCACAGG^{-162}) diverging by one bp from the published CP2 consensus, which has been defined by assessing the effect of in-frame clustered mutations within canonical, high-affinity CP2 sites, such as the proximal α-globin site or the γ-fibrinogen site, on RCP2 binding in EMSA (Lim et al., 1993). Although the nucleotide composition of the linker sequence only marginally affected CP2 binding in previous experiments, a 6-bp spacing of the two CNRG(C) boxes was found to be critical for the stability of the DNA-protein complex (Lim et al., 1993). Consistent with this view, we found that the *IL4* CPRE, featuring an imperfect distal CNRG box and a 5-bp linker, binds RCP2 with notably lower affinity than the proximal α-globin site.

Although a CPRE is apparently located within the proximal 175 bp of the *IL4* promoter, our findings suggest that additional nucleotide contacts outside of this region are necessary for full CP2 binding and function. We in fact show that: 1) RCP2 does not bind *in vitro* to an oligonucleotide spanning bp -175 to -146; 2) endogenous CP2 is not detectable within complexes forming on *IL4* promoter fragments extending through bp -175; and 3) deletion of the *IL4* promoter to bp -175 markedly decreases the transactivation potential of overexpressed CP2, residual transcriptional activation presumably due to the formation of low-stability complexes onto an incomplete site *in vivo*. In footprinting experiments using either Dnase I or $(OP)_2Cu^+$ as the cleaving agent — this latter allowing cleavage very close to the edge of the DNA sequence protected by protein binding — RCP2 protected a region extending through bp -177, *i.e.*, two nucleotides upstream of bp -175. Conceivably, these additional contacts, while not affecting the specificity of CP2 binding and function, might play a role in the stabilization of CP2-DNA complexes.

The *IL4* CPRE is surrounded by binding elements for the factors NFAT-1, CBF, IRF-2, STAT6, and HMG I(Y) (Szabo et al., 1993; Li-Weber et al., 1994; Klein-Hessling et al., 1996; Lederer

et al., 1996), suggesting that CP2 may interact with any of these factors. In particular, the 3' half of the CPRE partially overlaps the P2 sequence, which in a previous study of the mouse *IL4* promoter has been shown to interact with NFAT-1 or a related protein (Szabo et al., 1993). Indeed, native or bacterially expressed NFAT-1 can bind to a 175-146 oligonucleotide in EMSA. On the other hand, a CBF-binding Y/CCAAT box – the most distal of three similar functional elements identified in the *IL4* promoter – lies immediately upstream of the 5' end of the *IL4* CPRE (Figure 4A) (Li-Weber et al., 1994). Interestingly, CP2 and CBF/CP1 also bind to adjacent elements in the α-globin promoter, suggestive of possible interactions between the two factors (Kim et al., 1990). However, we have evidence that CP2 might not cooperate with CBF and/or NFAT-1 in this particular system. First, immunoreactive CP2 was undetectable in EMSA with nuclear extracts from Jurkat cells, expressing fair amounts of the protein, and an oligonucleotide probe (195-146) including the P2 and P3 NFAT-binding sequences and the distal CCAAT. In these experiments, a major complex was formed that contained CBF, as previously demonstrated in EMSA using a similar oligonucleotide (Li-Weber et al., 1994). Additionally, we have identified a complex of slower mobility, that apparently contained both CBF and NFAT-1. This was not unexpected, given the proximity of CBF and NFAT-1 recognition elements in this region of the *IL4* promoter, and the recent evidence that both factors contribute to the formation of the NF(P) complex on the human P1 sequence (Li-Weber et al., 1994). The addition of RCP2 in these experiments did not affect the mobility and/or the intensity of the CBF/NFAT-1 complexes, resulting in the formation of an additional complex of intermediate mobility. Second, CP2 does not physically interact with CBF (Zhong et al., 1994) or NFAT-1 in EMSA using shared oligonucleotides. Third, CP2 can repress NFAT-dependent transcription, as in the case of *IL2*.

The lack of detection of endogenous CP2 in EMSA using a 195-146 oligonucleotide was surprising. The binding conditions

for our experiments proved to be optimal for RCP2 binding to the same oligonucleotide and for the formation of a sequence-specific native complex on an α-globin consensus oligonucleotide. We obtained similar results using different nuclear extraction or binding conditions, including Ph, cation concentrations and the type of bulk carrier DNA used as nonspecific competitor. In contrast, consistent with our functional data, we detected immunoreactive CP2 in at least one constitutive complex forming in EMSA with a PCR-generated *IL4* promoter fragment extending to bp -225, but not with fragments lacking the region between bp -225 and -176. The same complex was not affected by anti-NFAT-1 antibodies, while two alternative NFAT-1-immunoreactive complexes were formed onto an *IL4*.175 fragment, which *in vivo* exhibited significantly lower constitutive and inducible activity than longer *IL4* deletional inserts. This stresses the role of endogenous CP2 in the assembly of a constitutive *IL4* transcriptional complex, while minimizing the contribution of NFAT-1 at least in this system.

Our findings suggest that the integrity of *IL4* promoter regions outside of the CPRE is required for optimal CP2 binding and function. This implies that CP2 must interact with other factors, not necessarily binding to contiguous elements, to result in the formation of a stable complex centered on a low-affinity site. In a previous study two classes of CPRE have been defined (Jane et al., 1995). The first, including the α-globin and γ-fibrinogen sites, preferentially binds CP2 as a homodimer, while second-class CPRE, including the γ-globin SSE and another site in the ε-globin promoter, bind CP2 as an obligate heterodimer, where stage- and/or lineage-specific cofactors possibly mediate CP2 binding and function (Jane et al., 1995). Although the *IL4* CPRE exhibits an imperfect half site, we cannot conclude that this site belongs to this second class of CP2 elements. Differently from a typical second class CPRE, the *IL4* element apparently binds rCP2 homodimers, the resulting complex

having identical mobility to that forming on an α-globin first-class site.

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure.

EXAMPLE 1

Identification of CP2 as a Transcriptional Activator of the Human *IL4* Gene

In previous studies potentially relevant *IL4* transcriptional regulators have been identified by assessing the effect of their overexpression or ectopic expression in cells transiently transfected with *IL4* promoter-driven reporter constructs. Among these, NFAT-1, c-Maf, C/EBPβ and NF-κB1 activate transcription from a panel of human or mouse *IL4* promoter constructs (Casolaro et al., 1995; Davydov et al., 1995; Ho et al., 1996; Luo et al., 1996), while other factors, such as RelA and HMG I(Y), exert a distinct inhibition of *IL4* promoter activity (Casolaro et al., 1995; Klein-Hessling et al., 1996). As an assay for *IL4* promoter activation, we analyzed the effect of CP2 overexpression on chloramphenicol acetyltransferase (CAT) gene expression driven by a region of the human *IL4* gene extending from bp -311 through +55 relative to the transcription initiation site. This region includes all the *IL4* promoter elements characterized to date in human or mouse T cells, and confers on heterologous reporter genes proper lineage- and activation-specific expression (Bruhn et al., 1993; Todd et al., 1993).

The human T cell line Jurkat has been widely used as a model for the study of human *IL4* transcription. These cells express a "Th0-like" phenotype, in that they are able to transcribe both the *IL2* and *IL4* genes (Arai et al., 1989). With respect to *IL4* transcription, Jurkat cells are in a preactivated state, characterized by constitutively elevated *IL4* promoter activity

and mRNA accumulation (Li-Weber et al., 1992). This may reflect dysregulation of the calcineurin-NFAT pathway in a leukemic cell line, although constitutive NFAT-1 nuclear translocation and *IL4* activation have also been observed in nontransformed Th2 clones (Lederer et al., 1994). Referring to Fig. 1, the effect of CP2 overexpression on *IL2* and *IL4* promoter activity in transiently transfected Jurkat cells was examined. The CAT reporter plasmids *IL2*-15ΔCX (*IL2*), bearing bp −319 to +52 of human *IL2*, or p*IL4*.311 (*IL4*), bearing bp −311 to +55 of human *IL4*, were transfected (1 μg each) in Jurkat cells along with 2 μg of Prc/CMV-CP2 expression plasmid (■) or a control pRc/CMV noncoding vector (□) as described in Materials and Methods. Cells were left uninduced or stimulated for 18 h with 20 ng/ml PMA and 1 *μM* A23187 as indicated. The absolute CAT concentration in cell lysates, normalized by total protein concentration, is indicated as the mean ± SEM of three independent transfections. We found that constitutive *IL4* promoter activity was enhanced up to five-fold in Jurkat cells overexpressing CP2, the degree of *IL4* transactivation by this factor being comparable to that obtained in cells overexpressing a constitutively active form of calcineurin. CP2 overexpression also resulted in significant, although less noticeable, increase of *IL4* promoter activity in cells stimulated with PMA (10 ng/ml) and Ca^{2+}-ionophore (A23187; 1 *μM*) or Ca^{2+} ionophore alone, which was sufficient to induce maximal *IL4* transactivation. This suggests CP2 direct involvement in the molecular pathways mediating *IL4* gene induction in these cells. In striking contrast, *IL2* promoter activity, which in Jurkat cells was only induced by stimulation with PMA and A23187, was not enhanced, but actually repressed, in CP2-overexpressing cells. Together, these data indicate that CP2 exerts opposite effects on *IL2* and *IL4* transcription.

EXAMPLE II

The Dimerization Domain of CP2 Is Necessary for IL4 Transactivation Characterization of a CP2 Dominant Negative

CP2 appears to represent a novel family of dimeric transcription factors binding direct DNA repeats. CP2 dimerizes in solution, and heterodimers of CP2 with other LBP-1 family members and nonrelated proteins have been described (Uv et al., 1994; Zhong et al., 1994; Jane et al., 1995). CP2 dimerization is not essential for DNA binding, as would be suggested by the dyad symmetry of the CP2 consensus recognition sequence, but it does affect the stability of CP2 nucleoprotein complexes (Uv et al., 1994; Zhong et al., 1994). The major CP2 dimerization domain has been located within a COOH-terminal region (aa 426 to 502) sharing significant homology to the *Drosophila* factor Elf-1 (Uv et al., 1994). To assess the functional impact of CP2 dimerization on *IL4* transactivation, we generated a series of CP2 COOH-terminal truncations and cloned them into a pRc/CMV vector for use in cotransfection experiments in Jurkat cells. Fig. 2A shows a schematic diagram of CP2 primary structure and of the structure of COOH-terminal truncations Δ1 through Δ9. The DNA-binding and dimerization domains, sharing homology to the *Drosophila* regulatory protein Elf-1, are indicated; a black box labelled "Elf-1" indicates the region encoded by exon 6 of the CP2 gene (aa 189-239), which is the most closely related to Elf-1 and is essential for DNA binding. Following a moderately basic region (▧) is a domain (SPXX; aa 250 to 405), rich in serine/threonine (17.5%) and proline (11%) residues, that contains several SPXX motifs. This domain is followed by a stretch of 10 glutamines or a sequence of alternating glutamine and proline residues (Q/P domain; ▨) in human or mouse CP2, respectively (Lim et al., 1992). The COOH-terminal dimerization domain is included in a region having a net moderately negative charge (net acidic). Also shown is a ΔElf-1 deletion (lacking aa 189-239). Fig. 2B shows that deletion of CP2 COOH-terminal 12

aa (Δ1) was sufficient to result in substantial loss of activity. However, only constructs lacking the COOH-terminal 77 aa (Δ3 through Δ9) were completely devoid of transcriptional activity. These data indicate that the CP2 dimerization domain is essential for CP2 function. Consistent with these findings, all of these truncations, expressed in bacteria as GST-fusion proteins, were shown, with the exception of Δ9, to specifically bind to a consensus CP2 site from the mouse α-globin promoter, while they were unable to dimerize with each other or with the full-length polypeptide (Zhong et al., 1994).

The symmetry of CP2 binding site, along with the factor's multimeric structure, suggested that it might be possible to generate dominant negative versions having a disrupted DNA binding, but intact dimerization domain. A possible CP2 dominant negative has been described as lacking the domain encoded by exon 6 (aa 189 to 239), which is the one most closely related to Elf-1 (Uv et al., 1994; Zhong et al., 1994). This protein, named in different studies LBP-1d, aCP2, or LSF-ID, is spontaneously generated in HeLa and other cells by alternative splicing or secondary splicing of CP2 transcripts and exhibits no DNA binding activity (Shirra et al., 1994; Uv et al., 1994; Yoon et al., 1994). In addition, it specifically reduced *in vitro* binding of full-length CP2 in a dominant manner (Zhong et al., 1994). However, the production *in vivo* of sufficiently high levels of LBP-1d to function as a dominant negative has not been demonstrated. We generated a protein identical to the previously described LBP-1d, which we will refer to as ΔElf-1 having the structure shown in Fig. 2A, to assess the role of DNA binding in CP2-mediated *IL4* promoter activation. Irrespective of the cell activation state, the results of Fig. 2C show that overexpression of the ΔElf-1 CP2 mutant strongly repressed *IL4* promoter activity in Jurkat cells. This result suggested that endogenous CP2, whose DNA binding is specifically reduced by this dominant negative antagonist, is an essential component of a constitutive *IL4* transcriptional complex.

EXAMPLE III

Identification of an *IL4* Promoter Region Necessary for CP2-mediated Activation

To map the *IL4* promoter region necessary for transactivation by CP2, we generated by PCR a series of deletional mutants for use in transfection studies. Fig. 3A shows a schematic representation of the *IL4* promoter and of PCR-generated deletional mutants bearing 5' deletions of the human *IL4* promoter to the indicated nucleotide upstream of the transcription initiation site. These fragments were inserted into pCAT-Basic to generate the reporter plasmids p*IL4*.741, p*IL4*.311, p*IL4*.265, p*IL4*.225, p*IL4*.175, p*IL4*.145, p*IL4*.95 and p*IL4*.65. Open and closed rectangles indicate the relative positions of positive and negative regulatory *cis* elements, respectively, identified to date in the human or mouse *IL4* promoter. Deletions were specifically designed to analyze the relative impact of *IL4* regulatory elements characterized to date on CP2-mediated activity. Positive regulatory elements include: the P0-P4 sequences, scattered throughout the promoter and shown to bind NFAT-1 and other factors, such as NF-κB (P1), CBF (P1), HMG I(Y) (P1), C/EBPβ (P4), and STAT6 (P2) (Szabo et al., 1993; Li-Weber et al., 1994; Casolaro et al., 1995; Davydov et al., 1995; Klein-Hessling et al., 1996; Lederer et al., 1996); two CCAAT/Y boxes, centered at bp -114 and -177, which also bind CBF (Szabo et al., 1993; Li-Weber et al., 1994); two octamer-associated protein (OAP) sites, located just upstream of the P1 sequence and in the context of the P4 sequence, which bind AP-1-related factors (Szabo et al., 1993; Rooney et al., 1995); and the c-Maf response element (MARE), located downstream of the P0 sequence and binding c-Maf and C/EBPβ (Davydov et al., 1995; Ho et al., 1996). Negative regulatory elements (NRE) include: NRE-I and NRE-II, contiguously located at bp -307, which bind as yet unidentified transcriptional repressor(s) (Li-Weber et al., 1992); an IRF-2-binding element (ISRE), located at bp -195 (Li-Weber et al., 1994); and an A/T-rich region starting at bp -209 and termed Box

II, presumably mediating repression by HMG I(Y) (Klein-Hessling et al., 1996).

As shown in Fig. 3B, transient transfection of Jurkat cells with CAT reporter plasmids bearing each of these *IL4* deletional mutants resulted in a consistent pattern of constitutive and induced CAT expression. In agreement with previous reports (Li-Weber et al., 1992), deletion of the NRE-I/NRE-II region, as achieved in constructs p*IL4*.265 and p*IL4*.225, resulted in significantly higher transcriptional activity than that seen with constructs p*IL4*.741 or p*IL4*.311. Both constitutive and induced *IL4* promoter activity significantly dropped upon deletion to bp -175, and was restored by further deletion to bp -145, suggesting the presence of an additional NRE in the region between bp -175 and -146. CP2 was more effective on promoter constructs truncated at bp -265 or -225 than on p*IL4*.311, presumably due to interference of negative regulation from the distal NREs. By contrast, removal of the region spanning bp -225 to -176 decreased transcriptional activation in CP2-overexpressing cells by almost four-fold, while constructs truncated at bp -145 through -65 were completely unresponsive to the factor. These data suggest that a CP2-recognition element (CPRE) is apparently located in the *IL4* promoter region spanning bp -175 to -146. However, additional nucleotides must be required upstream of bp -175 for full promoter inducibility by CP2.

EXAMPLE IV

Definition of a CPRE Within the *IL4* Promoter

The *IL4* promoter region included between bp -225 and -146 contains binding sites for numerous factors, including the P2 and P3 sequences (NFAT-1 and STAT6), a CCAAT box partially overlapping the P3 sequence (CBF), an ISRE (IRF-2) and the HMG I(Y)-binding A/T-rich Box II, as shown in Fig. 4A (Szabo et al., 1993; Li-Weber et al., 1994; Klein-Hessling et al., 1996; Lederer et al., 1996). However, in spite of a clear transcriptional effect, no obvious CP2 consensus element is recognizable in this

IL4 promoter region. To identify possible nonconsensus elements mediating CP2 binding and function within this region, we generated a series of partially overlapping oligonucleotides (as shown in Fig. 4A) for use as probes in electrophoretic mobility shift assay (EMSA). Referring to Fig. 4B, surprisingly, enriched, bacterially expressed CP2 (rCP2), used at a concentration sufficient for saturation of an α-globin consensus oligonucleotide (100 ng in 15 μl), did not bind to an oligonucleotide (175-146) including the *IL4* promoter region whose removal resulted in loss of CP2-mediated transactivation *in vivo* (Fig. 4B, lane 2). Additionally, rCP2 did not bind to oligonucleotide 225-176, spanning the *IL4* promoter region necessary for maximal transactivation (Fig. 4B, lane 4). These findings, in the light of our deletional analysis, suggested that nucleotide contacts in both regions might be necessary for CP2 binding. We therefore generated an oligonucleotide probe including the proximal 20 bp of the 225-176 region of the human *IL4* promoter in addition to the 175-146 region. Consistent with our hypothesis, this oligonucleotide (195-146) bound rCP2 consistently, yet much more weakly than the α-globin site (Fig. 4B, lane 3).

To define in detail a CPRE within this region of the human *IL4* promoter, we analyzed the pattern of nucleotide protection upon cleavage by copper-phenanthroline [$(OP)_2Cu^+$] of a 195-146 probe following EMSA with rCP2 (Sigman, 1990). Following EMSA with rCP2, free (F) and bound DNA (CP2) were subjected to cleavage within the gel matrix, then eluted and electrophoresed onto an 8% sequencing gel. Referring to Fig. 4C, the alignment with a Maxam-Gilbert G+A ladder of the same probe is shown. Numbers to the left indicate positions relative to the transcription initiation site. A hypersensitive site at bp -151 and a footprint extending from bp -158 to -177 are indicated to the right as an asterisk and a dashed vertical line, respectively. In these experiments rCP2 protected a DNA sequence extending from bp -177 to -158. An imperfect CPRE

($^{-174}$CTGATTTCACAGG^{-162}) is recognizable within this sequence. rCP2 only partially protected an adjacent CCAAT box ($^{-180}$ATTGG^{-176}), confirming that, in contrast to initial observations (Chodosh et al., 1988), CP2 is not a CCAAT-binding protein. The homology of the *IL4* promoter CPRE to other known CP2 sites within cellular and viral promoters is indicated in Fig. 4D, which shows the alignment of the *IL4* promoter region protected by rCP2 with a series of CPRE identified in some cellular and viral promoters. Differently from most CP2 elements, a conserved CNRG box in the *IL4* sequence ($^{-165}$CAGG^{-162}) is separated from an imperfect one ($^{-174}$CTGA^{-171}) by a 5-bp linker. It has been reported that CNRG repeats separated by 5 bp, such as the α-globin distal CPRE, bind CP2 with about four-fold lower affinity than elements containing a linker of 6 bp (Lim et al., 1993). These divergences might explain the affinity gap between the *IL4* and α-globin oligonucleotides used in this study.

EXAMPLE V

CP2 Expression in Jurkat Cells Is Not Paralleled by CP2 Complex Formation on an *IL4* CPRE Oligonucleotide

Our findings in Jurkat cells overexpressing the ΔElf-1 dominant negative of CP2 suggest that endogenous CP2 is a critical component of a constitutive transcriptional complex forming on the human *IL4* promoter in these cells. Referring to Fig. 5A, binding of Jurkat nuclear proteins (5 μg) to a 5′ end-labeled α-globin (lanes 1–5) or 195–146 oligonucleotides (lanes 6–10) was examined. Following incubation with the DNA probes, extracts were incubated with rabbit preimmune serum (PS; lanes 1 and 6) or the indicated antibodies (1 μg each). Complexes containing immunoreactive CP2 (lane 2), NFAT-1 and/or CBF-A are indicated. Also shown are four additional complexes (I–IV) forming on the 195–146 probe. The results of these experiments show that a CP2-immunoreactive complex is formed on a mouse α-globin CP2 oligonucleotide in EMSA with nuclear extracts from unstimulated Jurkat cells. We also found that CP2 expression

and/or function in these cells was approximately three-fold lower than in HeLa or K562 cells, and was almost unaffected by cell treatment with PMA and A23187. Several complexes were detectable in EMSA with Jurkat nuclear extracts and an oligonucleotide probe (195-146) including the CPRE within the human *IL4* promoter. Surprisingly, the formation of none of these complexes was affected by coincubation with a rabbit anti-CP2 antiserum (lane 7), while at least two complexes appeared to contain CBF-A (NF-YB) (lane 8), in substantial agreement with previous findings (Li-Weber et al., 1994). The slower-migrating of these two complexes was also reactive to a specific anti-NFAT-1 antibody (lane 10), consistent with the presence, in this *IL4* promoter region, of two potential NFAT-1 recognition sites (the P2 and P3 sequences), and with the possible formation of CBF-NFAT-1 complexes, as previously proposed (Li-Weber et al., 1994). Factors other than NFAT-1 or CBF seem to account for the formation of the faster-mobility complexes I-IV. Two of these complexes presumably correspond to the B2 and B3 complexes previously detected on a 195-163 oligonucleotide, which have been found to contain IRF-2 and an NF-1-like factor, respectively (Li-Weber et al., 1994).

Thus, the binding of endogenous CP2 to a low-affinity CPRE within the *IL4* promoter is undetectable under *in vitro* experimental conditions that are favorable for rCP2 binding to the same site and for formation of a CP2-immunoreactive native complex onto a canonical α-globin element. This is presumably due to the lability of CP2 low-affinity interaction with its cognate site and/or possible heteromeric partners. Referring to Fig. 5B, the addition of rCP2 (100 ng) to crude extracts in these experiments resulted in the formation of a complex of intermediate mobility between the CBF and the CBF-NFAT-1 complexes. This complex migrated at a significantly slower mobility than the complex formed with rCP2 alone, suggesting that endogenous proteins might contribute to its formation. These might not include NFAT-1 or CBF, since rCP2 did not apparently

dimerize with native CBF (Zhong et al., 1994) or with bacterially expressed NFAT-1. Complex I did not form in samples containing rCP2 (compare lanes 2 and 3). The nature and the sequence-specificity of this complex were, however, unclear, since competition with a comprehensive panel of unlabelled heterologous oligonucleotides did not result in its ablation.

EXAMPLE VI

Detection of a CP2 Immunoreactive Complex Forming onto Full-length Fragments of the Human *IL4* Promoter

Taken together, these findings led us to speculate that additional nucleotide contacts outside of the 195-146 region might be necessary for preferential binding of CP2 to an *IL4* low-affinity CPRE in Jurkat cells. This prompted us to investigate whether CP2 contributes to the formation of transcriptional complexes forming onto more comprehensive regions of the human *IL4* promoter. We therefore generated by PCR of a p*IL4*.265 template a panel of ^{32}P end-labelled deletional fragments of the *IL4* promoter for use in EMSA or footprinting experiments with rCP2 or Jurkat nuclear extracts. Referring to Fig. 6A, in DNase I footprinting experiments using an end-labelled DNA fragment spanning bp -265 to +55 of human *IL4*, binding of rCP2 resulted in the appearance of a weak footprint corresponding to the region included between bp -177 and -150, in agreement with our previous analysis. This was paralleled by increased sensitivity to cleavage by DNase I of a region extending upstream of bp -180, which was clearly dependent on the amount of rCP2 used. No other nucleotide contacted by rCP2 was found in any region of the *IL4* promoter in these experiments, while the whole DNA fragment was extensively protected by nuclear proteins extracted from unstimulated Jurkat cells (lane 3). These findings suggest that the CPRE that we previously located between bp -177 and -158 is the only *IL4* promoter site interacting with CP2 that might account for the factor's transcriptional effect. Although this region was protected in experiments with Jurkat extracts, we

could not determine whether endogenous CP2 or factors binding to partially overlapping sites (*i.e.*, NFAT-1 and/or CBF) might account for this finding.

To elucidate this point, we analyzed by EMSA with Jurkat nuclear extracts the pattern and composition of complexes forming on serial deletions of a region of human *IL4* extending through bp +55. The results are shown in Fig. 6B. We found that a major constitutive complex forming onto a full-length *IL4* promoter fragment spanning bp -225 to +55 (*IL4*.225) was diminished by coincubation with a rabbit anti-CP2 antibody (middle lane), while a specific anti-NFAT-1 antibody did not have any effect (right lane). A similar complex did not appear in EMSA with *IL4* promoter fragments lacking the 225-175 region (*IL4*.175 through *IL4*.65), suggesting that the formation of this CP2-immunoreactive complex requires critical nucleotide contacts within this region. Surprisingly, NFAT-1, reported to interact with at least four sites within the proximal 225 bp of the *IL4* promoter (Szabo et al., 1993), is not apparently a component of this complex. NFAT-1-immunoreactive complexes were instead detected on the *IL4*.175 and *IL4*.95 fragments, suggesting that the exposure of different regions of the *IL4* promoter leads to the formation of alternative transcriptional complexes of different composition and perhaps function.

Consistent with these data and with our EMSA findings (refer again to Fig. 4B), rCP2 bound only to *IL4* promoter fragments extending upstream of bp -175. Referring to Fig. 6C, both rCP2 binding and the formation of a CP2-immunoreactive native complex on an *IL4*.225 probe were specifically inhibited by competition with a 50-fold molar excess of unlabelled 195-146 oligonucleotide, but not by oligonucleotides spanning other regions of the human *IL4* promoter, consistent with our footprinting analysis. Together, these results suggest that the architecture of the entire promoter is necessary to reconstitute a high-affinity CP2-binding site *in vitro* and perhaps *in vivo*. This, rather than providing nucleotide contacts for CP2 itself

in addition to the CPRE, presumably acts by favoring the interaction with other factors resulting in the stabilization of CP2 binding to an otherwise low-affinity site.

Materials and Methods

Plasmid Construction. The *IL4* promoter fragments used in this study were initially generated by PCR using human genomic DNA as a template and cloned into the *Hin*dIII and *Xba*I sites of a pBluescript vector (Stratagene Cloning Systems, La Jolla, CA). In each case, an *Xba*I-tailed oligonucleotide, corresponding to bp +36 to +55 of human *IL-4* and one of four *Hin*dIII-tailed oligonucleotides, corresponding to bp -741 to -722, -311 to -292, -265 to -246, -225 to -206, -175 to -156, -145 to -126, -95 to -76, or -65 to -46, were used to introduce the appropriate restriction sites at the invariant 3′ and at the 5′ ends, respectively. After sequence verification, each fragment was inserted into the *Hin*dIII and *Xba*I sites of the pCAT-Basic vector (Promega Corporation, Madison, WI) to construct the corresponding reporter plasmids p*IL4*.741, p*IL4*.311, p*IL4*.265, p*IL4*.225, p*IL4*.175, p*IL4*.145, p*IL4*.95 and p*IL4*.65. The CP2 expression plasmid (in pRc/CMV; Invitrogen Corporation, San Diego, CA) has been described in previous studies (Lim et al., 1993). COOH-terminal truncations of the full-length polypeptide and the Elf-1 deletion were prepared by PCR as described (Zhong et al., 1994), and inserted into the same CMV-driven vector. The *IL2*.15ΔCX reporter plasmid, bearing bp -319 to +52 of human *IL2* (Shaw et al., 1988), has been kindly donated by Dr. G. R. Crabtree (Stanford University, Stanford, CA).

Cell Cultures and Transfections. A line of Jurkat T cells, constitutively expressing *IL4* and producing *IL2* following activation, was maintained in RPMI 1640, 25 m*M* HEPES, 2 m*M* L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), and 50 μg/ml gentamicin. Aliquots of cells frozen at early passages were recovered from liquid nitrogen bimonthly, and used for

experiments between 1 and 5 wk after thawing. Cells (3×10^6) were transfected with 1 μg CAT reporter plasmid and 2 μg expression plasmids by 48-h culture in RPMI 1640 containing 5.2 mg/ml Lipofectamine (Life Technologies, Gaithersburg, MD), according to the manufacturer's specifications (Casolaro et al., 1995). Equal amounts of the corresponding noncoding vectors were added to control samples to yield a constant amount (3 μg) of DNA in each transfection. Where indicated, cells were stimulated 16-18 h before harvest. For CAT determination, 40-80 μg of total cellular proteins from each sample, extracted by lysis in detergent solutions and measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA), were diluted to a total of 200 μl for analysis using a commercially available sandwich ELISA kit (Boehringer Mannheim, Indianapolis, IN). CAT concentrations have been expressed as pg/μg total protein.

Nuclear Extracts and Recombinant Proteins. Nuclear extracts have been prepared by a modification of a described protocol (Li et al., 1991). Cells (5×10^7) were allowed to swell in 10 m*M* HEPES, pH 7.9, 30 m*M* KCl, 1 m*M* dithiothreitol (DTT), 0.1 m*M* EDTA, 0.1 m*M* EGTA, 0.5 m*M* phenyl methyl sulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin, and 1 μg/ml aprotinin, then lysed by the addition of 0.075% NP40. Nuclei were separated from the cytosolic extract by centrifugation for 4 min at 3,000 rpm in microfuge, resuspended in 20 m*M* HEPES, pH 7.9, 420 m*M* KCl, 1 m*M* DTT, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 0.5 m*M* PMSF, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, and 20% glycerol, and left on ice for 40 min. Nuclear debris and membranes were pelleted for 10 min at 14,000 rpm in microfuge. The supernatant was removed, aliquoted, quick-frozen in liquid nitrogen, and stored at -80°C. Protein concentrations in all extracts were measured by the Bradford method (Bio-Rad). Bacterially expressed CP2 was prepared and enriched as described previously (Lim et al., 1993).

Oligonucleotides and *IL4* Promoter Fragments. The following oligonucleotides and their complements were synthesized: 5'-TGCTGAAACTTTGTAGTTAATTTTTTAAAAAGGTTTCATTTTCCTATTGG-3'(225-176), 5'-AGGTTTCATTTTCCTATTGGTCTGATTTCACAGGAACATTTTACCTGTTT-3' (195-146), 5'-TCTGATTTCACAGGAACATTTTACCTGTTT-3' (175-146), 5'-GTGAGGCATTTTTTCTCCTGGAAGAGAGGTGCTGATTGGCCCCAAGTGAC-3' (145-96), 5'-TGACAATCTGGTGTAACGAAAATTTCCAATGTAAAC-3' (95-60), and 5'-GATCCCAAGTTTTACTGGGTAGAGCAAGCACAAACCAGG-3' (α-globin CP2). Double-stranded oligonucleotides were 5'-end-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) using γ-^{32}P-ATP (Amersham Corporation, Arlington Heights, IL) and purified by electrophoresis on 4% polyacrylamide gels. For some experiments, larger regions of the human *IL4* promoter, corresponding to the deletional fragments inserted into CAT vectors, were generated by PCR of a p*IL4*.741 template using the *Xba*I and *Hin*dIII primers described in an earlier section. These fragments were labeled by inclusion of either 5' end-labeled primers or α-^{32}P-dCTP in the PCR reaction.

EMSA. Probes (10,000-30,000 c.p.m., corresponding to 5-20 fmol) were incubated (20-30 min, 25°C) with 1-5 μg nuclear extracts or 10-100 ng rCP2 in 15 μl of 12 m*M* Hepes, pH 7.9, 50 m*M* KCl, 0.5 m*M* $MgCl_2$, 0.12 m*M* EDTA, 0.12 m*M* EGTA, 4 m*M* DTT, 0.1% NP40, 12% glycerol, 0.1 mg/ml bovine serum albumin (BSA) and 30 μg/ml (10 μg/ml in the case of rCP2) poly(dI·dC) (Pharmacia Biotech Inc., Piscataway, NJ). Where indicated, before addition of the probe, extracts were incubated 10 min at 25°C with a 50-fold molar excess of competitor unlabelled oligonucleotides. Alternatively, 20 min after addition of the probe, the binding reactions were incubated 30 min at 4°C with rabbit antisera specific for the transcription factors CP2, NFAT-1 (Upstate Biotechnology, Lake Placid, NY), CBF-A (Accurate Chemical and Scientific Corp., Westbury, NY), HMG I(Y) (kindly provided by Dr. D. Thanos, Columbia University, New York, NY). This treatment led, under the experimental conditions described, to specific

ablation of the immunoreactive complexes, with almost no detectable "supershift". Free probes and DNA-protein complexes were resolved by electrophoresis on 4% native polyacrylamide gels in 45 m*M* Tris, pH 8.2, 45 m*M* boric acid, 1 m*M* EDTA and 1% glycerol and visualized by autoradiography of fixed and dried gels.

Footprinting of the Human *IL4* Promoter. DNase I and $(OP)_2Cu^+$ footprinting experiments were performed as described (Sigman, 1990; Lim et al., 1993). To examine the binding of rCP2 to the *IL4* promoter, the *Xba*I-*Hin*dIII insert of a p*IL4*.265 plasmid, labeled by filling of either 3′ end with α-^{32}P-dCTP using the Klenow enzyme, or a 195-146 oligonucleotide 5′ end-labeled on the coding or noncoding strand, were incubated (30 min, 25°C) with the indicated amounts of nuclear extracts or rCP2 in 25 μl of EMSA buffer (described above). For DNase I cleavage, $MgCl_2$ was added to 5 m*M*, then samples were digested with DNase I (100-200 ng for nuclear extracts, 20-50 ng for rCP2, and 10 ng for control reactions) for 1 min at 25°C. Alternatively, for treatment with $(OP)_2Cu^+$, samples were electrophoresed onto a 4% polyacrylamide gel, which was then immersed into a solution containing 10 m*M* Tris, pH 8.0, 0.2 m*M* 1,10-phenanthroline and 0.045 m*M* $CuSO_4$. The chemical nuclease reaction was started by addition of mercaptopropionic acid to 0.05% and allowed to proceed for 12 min at 25°C, then quenched in 2 m*M* 2,9-dimethyl-1,10-phenanthroline. Free DNA and DNA-protein complexes, visualized by autoradiography, were eluted (18 h at 37°C) from the gel matrix in 0.5 *M* ammonium acetate, pH 7.5, 1 m*M* EDTA, and 0.1% sodium dodecyl sulfate (SDS). Equivalent amounts of DNA from each sample and of a Maxam-Gilbert G+A ladder of the same probes were resolved onto an 8% acrylamide/7 *M* urea gel.

Use

As CP2 has now been shown to regulate both *IL4* and *IL2* expression, interruption or enhancement of CP2 activity and thus regulation of Th1/Th2 cell balance, can be used for therapeutic control of the immune response and immunologic disease in a variety of conditions. These include, but are not restricted to: allergic rhinitis, allergic conjunctivitis, asthma, dermatitis, urticaria, multiple sclerosis, type I diabetes mellitus, arthritis and parasitic infection. CP2 or dominant negative CP2 may also be useful in the management of immunodeficiency disorders or malignancies by amplifying T helper cell responses to viral antigen.

There is now abundant evidence that the nature of T helper cell response to autoantigen or foreign antigen plays an important part in disease onset and/or severity. In insulin dependent diabetes mellitus, Ono and coworkers first showed that interferon-gamma mRNA is found in the pancreas at very early stages of insulitis (Ono et al., 1988). Rabinovitch and coworkers confirmed this finding, and showed that expression of this and other Th1 cytokines correlates with beta-cell destruction in BB rats (Rabinovitch et al., 1996). Additional evidence for a role for Th1 cells in IDDM came from the laboratory of Adorini (Trembleau et al., 1995), where interleukin 12 administration accelerated the onset of diabetes in the NOD mouse. However, the nature of the response is certainly complex, as production of *IL10* by islet cells in transgenic mice also accelerated immune-mediated destruction of beta cells in NOD mice (Wogensen et al., 1994). Taken together, these data indicate that while spontaneous IDDM may usually result from a Th1 mediated phenomenon, the balance of the Th1/Th2 response may be the critical issue in determining disease susceptibility.

Several other autoimmune diseases also appear to depend upon the nature of the T helper cell response to autoantigen. Multiple sclerosis and its experimental model (experimental allergic encephalomyelitis (EAE)) also appears to be a Th1

mediated disease. Most T cell lines which are capable of inducing disease in animal models are Th1 cells, and *IL4* treatment results in induction of autoantigen-specific Th2 cells, diminished demyelination and significant amelioration of clinical disease (Cua et al., 1995). Direct analysis of cytokine mRNAs in inflamed joints from patients with rheumatoid arthritis indicates that a Th1 response predominates, whereas in reactive arthritis, both the Th1 cytokine, interferon-gamma, and the Th2 cytokine, *IL4*, are expressed (Simon et al., 1994). Finally, a similar analysis of local cytokine production in salivary gland biopsies from Sjögren's patients indicates that CD4+ T cells from these patients produce over 40-fold more *IL2*, IFN-gamma, and *IL10* mRNA than peripheral blood CD4+ T cells, and express little or no *IL4* and *IL5* mRNA (Fox et al., 1994).

In contrast to these observations with organ-specific autoimmune diseases, allergic diseases show a strong correlation with Th2 responses. Nasal biopsies from adult patients with seasonal allergic rhinitis or conjunctivitis exhibit elevated levels of mRNA for the Th2 cytokines, *IL2*, *IL4*, and *IL5*, and little or no Th1 cytokine gene expression (Karlsson et al., 1995). Very similar findings are found in allergic asthmatics. Analysis of late phase allergic asthmatics typically show recruitment of eosinophils to the airways, *IL4* mRNA in BAL fluids, and Th2 cytokines by ELISA (Bell et al., 1996). In summary, an elevated Th1 response appears to be critical for autoimmune diseases, while an elevated Th2 response appears to be critical for allergic diseases.

Existing methods of controlling such conditions rely primarily on pharmaceuticals such as steroids, with all their associated negative side effects, or (in the case of allergic diseases) on inhibitors of disease mediators. Other known methods of immunomodulation rely upon humanized monoclonal antibodies.

In contrast, in the methods of the invention, immunomodulation, i.e., regulation of Th1/Th2 cell balance through therapeutic control of CP2 activity, can be accomplished by gene therapy techniques or by administration of small molecule inhibitors or activators of CP2/*IL4* promoter binding. Treatment of tumors and other diseases in animal models by gene therapy has been demonstrated and can be carried out with CP2 gene based mammalian expression vectors, particularly vectors under the control of a T cell-specific promoter. For example, Nishimura et al. were able to apply *IL12* antitumor gene therapy to B lymphoma cells (Nishimura et al., 1996). Tumor cells transfected with both the B7-1 and *IL12* genes almost completely lost their metastatic potential. More recently, these methods have been extended for the treatment of human tumors (Tan et al., 1996). Retroviral mediated transfer of the *IL2* gene into tumor infiltrating lymphocytes was shown to have minimal side effects when re-infused into the chest cavity of patients in a Phase I clinical trial, and some of the patients showed resolution of pleural effusions and decrease in tumor burden. Thus, the methods for transfer of CP2 based genes into lymphocytes and their reintroduction into patients are in the public domain. Moreover, these methods have been shown to be safe and can be efficacious. In another example, *IL10* gene vectors were successfully transferred into murine cardiac allografts (Qin et al., 1996). Once again, retroviral mediated gene transfer was used to direct *IL10* gene expression in donor tissue. Without conventional immunosuppression, cardiac allograft survival was significantly prolonged in mice receiving the cytokine gene therapy. Finally, adenovirus mediated gene therapy is also a viable alternative mood. Replication-deficient recombinant adenovirus has been routinely used to transfer a variety of genes into most types of human tissue (Wilson et al., 1995; Goebel et al., 1996). CP2 expression vectors can also be administered locally, e.g., near the ocular surface, so that transfection can take place *in vivo*.

The search for small molecules that may interfere with endogenous CP2, and therefore endogenous cytokine gene transcription, can be carried out using existing or newly prepared small molecule libraries, which can be tested in high throughput screens. One such screen is an electrophoretic gel mobility shift assay in which recombinant CP2 bound to the CP2 recognition element (CPRE) described herein is resolved from unbound CPRE on nondenaturing acrylamide gels. The complex runs more slowly through the gel than the free probe (radiolabeled CPRE). The screen for compounds that inhibit CP2 interaction with the recognition element would involve the addition of physiologic concentrations of each compound to separate binding reactions. An effective competitor would abolish CP2/CPRE complex formation, which is easily seen on the mobility shift experiment. Compounds testing positive in high throughput screens are retested in a more sensitive secondary screen, such as the Jurkat cell assay system described herein. If compounds in known small molecule libraries fail to inhibit CP2/CPRE interaction, peptides derived from the CP2 DNA-binding domain or other small molecules, can also be tested for inhibitory capacity. Such peptides can be chosen randomly as overlapping peptides (LeSauteur et al., 1995; Digard et al., 1995), or after rational drug design, by methods well known to those of skill in the art. For example, peptides corresponding to the DNA binding domain of CP2 (SEQ ID NO: 2) or to the Elf-1 domain (SEQ ID NO: 3) within the DNA binding domain, or to fragments thereof of 10 amino acid residues or greater, are likely to be effective competitors to CP2/CPRE complex formation.

Oligonucleotides that might interfere with CP2/CPRE interaction include those that are homologous to the CPRE region itself as shown in Fig. 4D. Such oligonucleotides include

5'GTCTGATTTCA-CAGGAA3' (SEQ ID NO: 4);

5'AACAAGTTTTA-CTGGGT3' (SEQ ID NO: 5);

5'AGCAAGCACAAACCAGCC3' (SEQ ID NO: 6);

5'GACCAGTTCCAGCCACTC3' (SEQ ID NO: 7);

5'TACTGGGTCTCTCTGGTT3' (SEQ ID NO: 8);
5'TTCTGCCTCAGTCTGCGA3' (SEQ ID NO: 9);
5'TCCTCCCTATTCCGGGCC3' (SEQ ID NO: 10);
or generically: 5'CNRGNNNNNNCNRG3' (SEQ ID NO: 11).

It is well known that oligonucleotides can effectively permeate cell and nuclear membranes; therefore, the compounds described above, and modifications and derivatives thereof, can be expected to interfere effectively with CP2/CPRE interaction *in vivo*. For example, modifications to improve nuclease resistance and cellular uptake, such as incorporating phosphorothiote or phosphonodithioate residues into the phosphodiester backbone, are disclosed in U.S. Pat. Nos. 5,378,825 and 5,194,599, hereby incorporated by reference herein. Other common modifications to enable a compound to be used as a therapeutic agent include alkylation and glycosylation. An oligonucleotide used in the therapeutic composition of the invention is preferably 20-100 residues in length.

Alternatively, antisense oligonucleotides and derivatives thereof complementary to the sequence:
5'cctggggcaa ggaaggagcc aggatggcct gggctctgaa gctgcctctg3' (SEQ ID NO: 12) would be expected to interfere with CP2 production by inhibiting translation of the CP2 mRNA by ribosomes (Alama et al., 1997).

The therapeutic methods described herein may be tested for effectiveness in the various animal models that have now been developed for specific human diseases. For example, the NOD mouse is a well characterized model of type I diabetes mellitus where the T helper cell response is critical (Hultgren et al., 1996). The EAE model is an excellent model of multiple sclerosis (Krakowski et al., 1996), and there are now several animal models of asthma (Corry et al., 1996).

Modulation of *IL4* production has been shown to be linked in animal models to treatment of both allergic and autoimmune diseases (Kuchroo et al., 1995; Wilson et al., 1998). Therefore, compounds shown to be effective in the various animal models

described above are likely to be effective for therapeutic control of immune response and immunological disease in humans.

The therapeutic compositions including agents to interrupt or enhance CP2 activity for treatment of conditions such as those described above may be administered orally, topically, or parenterally, (e.g., intranasally, subcutaneously, intramuscularly, intravenously, or intra-arterially) by routine methods in pharmaceutically acceptable inert carrier substances. Topical administration at an affected site is preferred. For example, the therapeutic compositions of the invention, useful in the methods of the invention, may be administered by on-site delivery using micelles, gels or liposomes. A sustained release formulation using a biodegradable biocompatible polymer is preferred. The therapeutic agents can be administered in a dosage of 0.25 μg/kg/day to 5 mg/kg/day. Optimal dosage and modes of administration can readily be determined by conventional protocols.

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CLAIMS

What is claimed is:

1. A therapeutic composition comprising a therapeutically effective amount of an affector of CP2 function in a pharmaceutically acceptable carrier substance.

2. The therapeutic composition of claim 1 wherein said affector of CP2 function is an inhibitor of CP2 function.

3. The therapeutic composition of claim 1 wherein said affector of CP2 function is an activator of CP2 function.

4. The therapeutic composition of claim 1 wherein said affector of CP2 function is an affector of CP2 function.

5. The therapeutic composition of claim 1 wherein said affector of CP2 function is an affector of complex formation between CP2 and a CP2 recognition element in the human IL4 promoter.

6. The therapeutic composition of claim 1 wherein said affector is a compound identified using the method of claim 15.

7. The therapeutic composition of claim 2 wherein said inhibitor is a peptide selected from the group consisting of CP2ΔElf-1, a peptide having the sequence of SEQ ID NO: 1, a peptide having the sequence of SEQ ID NO: 2, and a peptide having the sequence of SEQ ID NO: 3.

8. The therapeutic composition of claim 2 wherein said inhibitor is an oligonucleotide selected from the group consisting of SEQ ID NO: 4 - SEQ ID NO: 11.

9. A method of treating a mammal to decrease an inflammatory response comprising administering to said patient a therapeutically effective amount of an inhibitor of CP2 function in a pharmaceutically acceptable carrier substance.

10. A method of treating a mammal to increase an inflammatory response comprising administering to said patient a therapeutically effective amount of an activator of CP2 function in a pharmaceutically acceptable carrier substance.

11. A method of treating a mammal to decrease an inflammatory response, said method comprising

providing T lymphocytes from said mammal;

transfecting said T lymphocytes with a mammalian expression vector comprising a CP2 cDNA sequence; and

administering said transfected T lymphocytes to said mammal in a therapeutically effective amount to decrease said inflammatory response.

12. A method of treating a mammal to increase an inflammatory response, said method comprising

providing T lymphocytes from said mammal;

transfecting said T lymphocytes with a mammalian expression vector comprising a CP2 cDNA sequence having a deletion in the DNA binding domain or dimerization domain; and

administering said transformed T lymphocytes to said mammal in a therapeutically effective amount to increase said inflammatory response.

13. A method of treating a mammal to decrease an inflammatory response, said method comprising

identifying an area of said mammal having a local inflammatory response; and

administrating a therapeutic composition comprising a mammalian expression vector, said vector comprising a CP2 cDNA

sequence, to said area of local inflammatory response, whereby T lymphocytes in said area of local inflammatory response are transfected with said expression vector in a therapeutically effective amount to decrease said inflammatory response.

14. A method of treating a mammal to increase an inflammatory response, said method comprising

identifying an area of said mammal in need of a local inflammatory response; and

administrating a therapeutic composition comprising a mammalian expression vector, said vector comprising a CP2 cDNA sequence having a deletion in the DNA binding domain or dimerization domain of said sequence, to said area in need of a local inflammatory response, whereby T lymphocytes in said area in need of a local inflammatory response are transfected with said expression vector in a therapeutically effective amount to increase said inflammatory response.

15. A method of screening for an affector of CP2 function, said method comprising the steps of:

providing first and second samples of components for an assay for complex formation between CP2 and a CP2 recognition element in the human IL4 promoter;

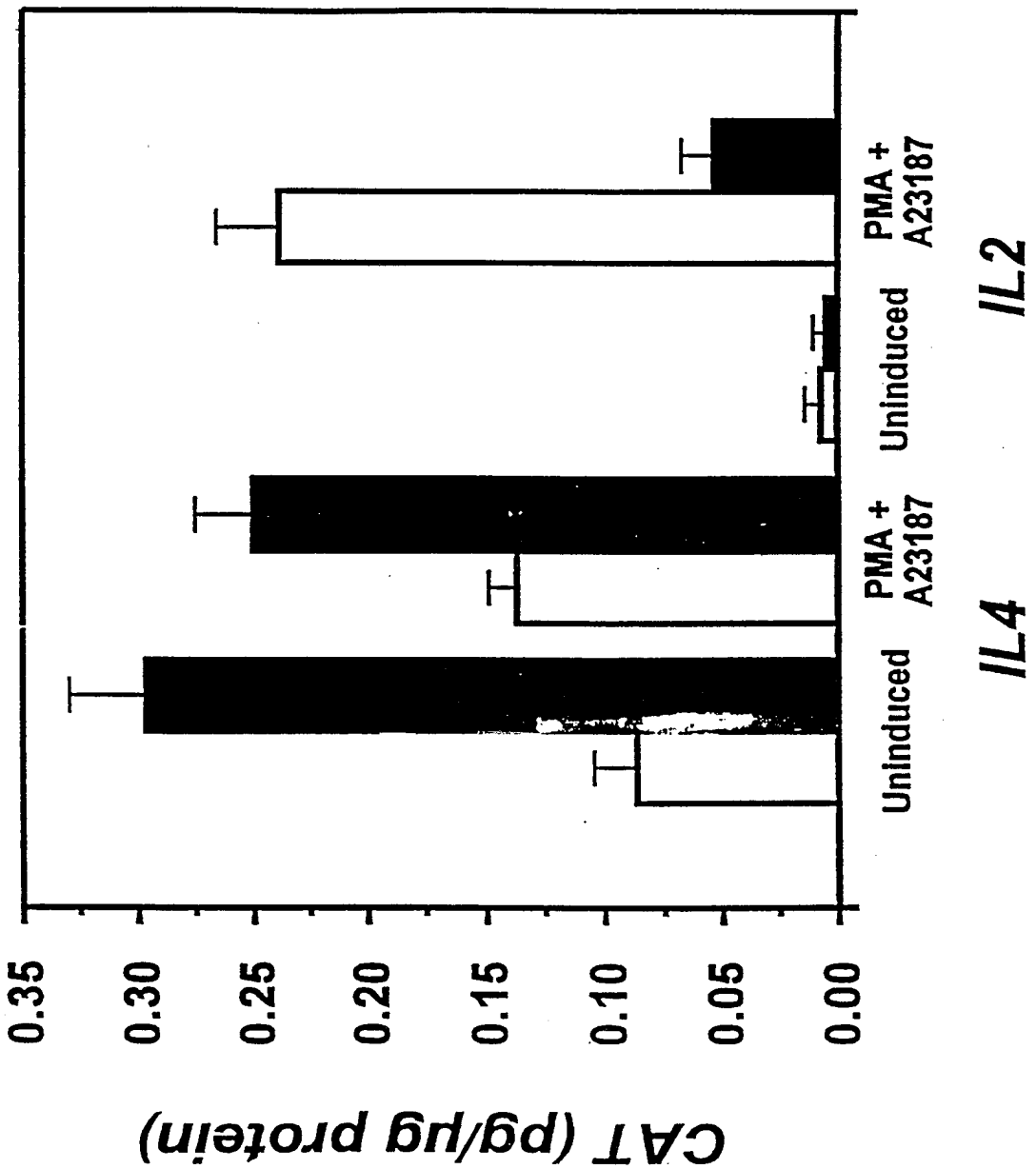
causing said first sample of components to react in said assay, wherein the extent of complex formation between CP2 and a CP2 recognition element in the human IL4 promoter in said first assay sample is determined;

adding a candidate affector to said second sample of components;

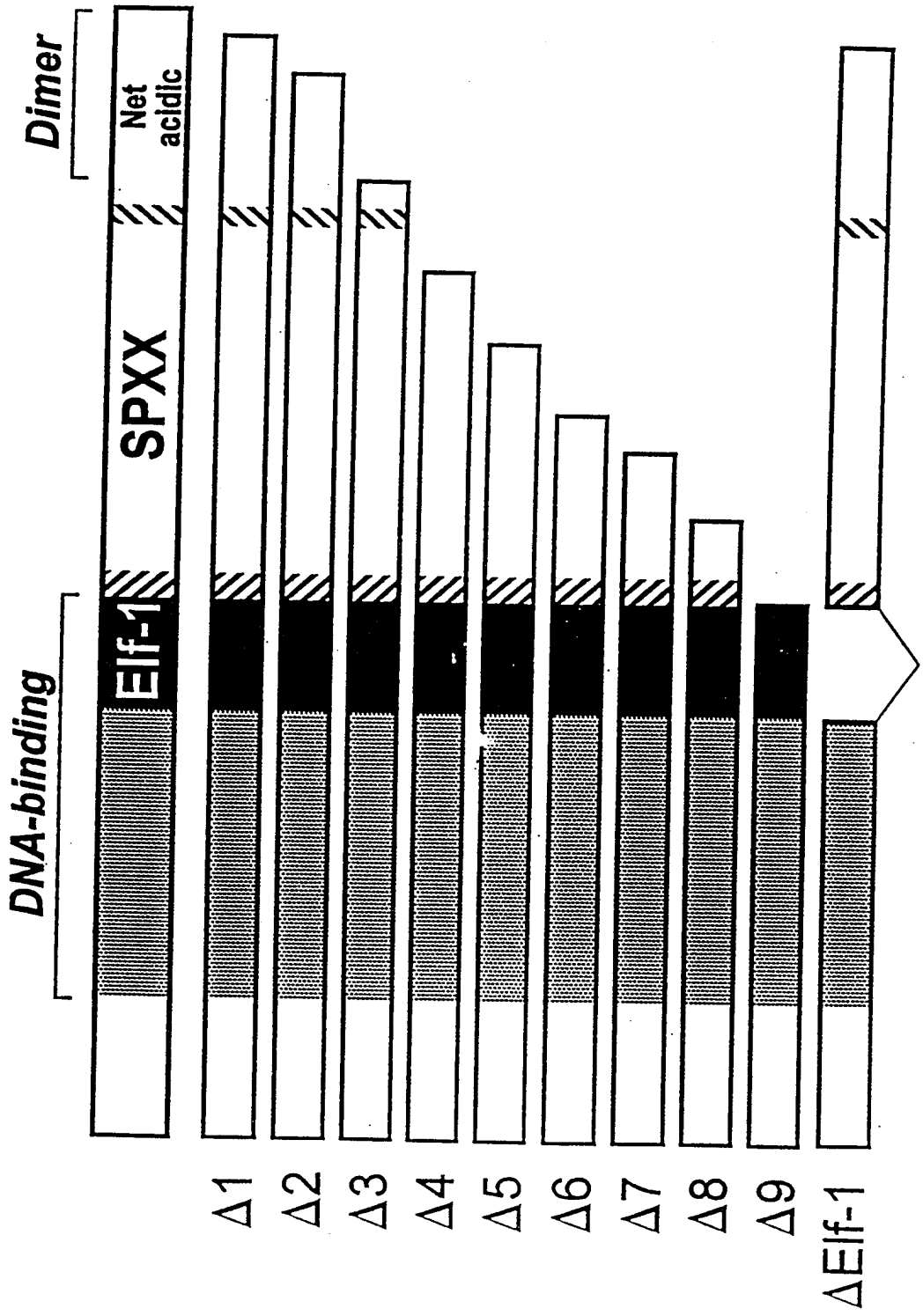
causing said second sample of components containing said candidate affector to react in said assay, wherein the extent of complex formation between CP2 and a CP2 recognition element in the human IL4 promoter in said second assay sample is determined; and

comparing said extent of complex formation between CP2 and a CP2 recognition element in the human IL4 promoter in said first assay sample to said extent of complex formation between CP2 and a CP2 recognition element in the human IL4 promoter in said second assay sample to determine the effect of said candidate affector.

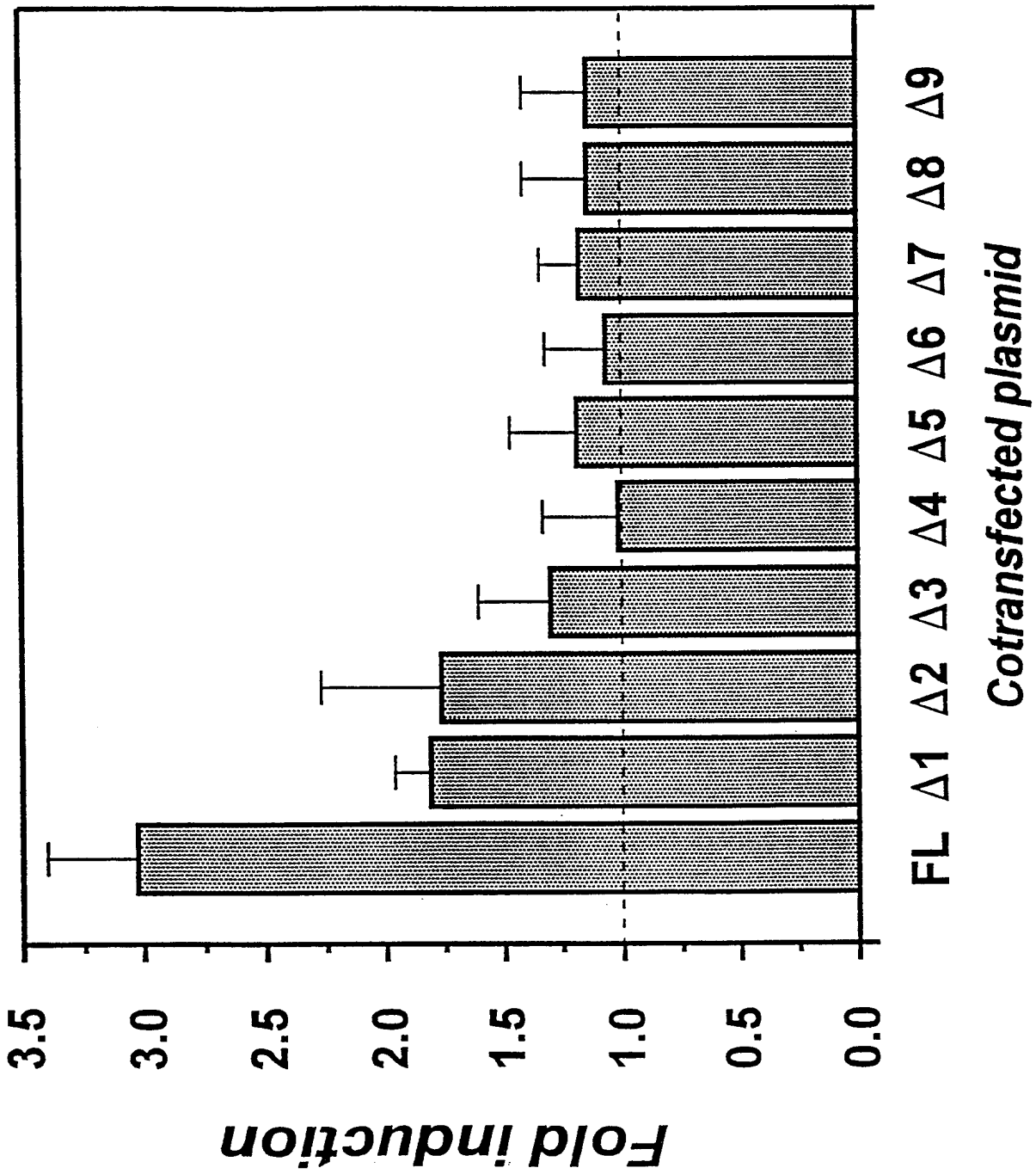
— Fig. 1


CAT (pg/µg protein)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Uninduced
PMA + A23187
Uninduced
PMA + A23187
IL4
IL2

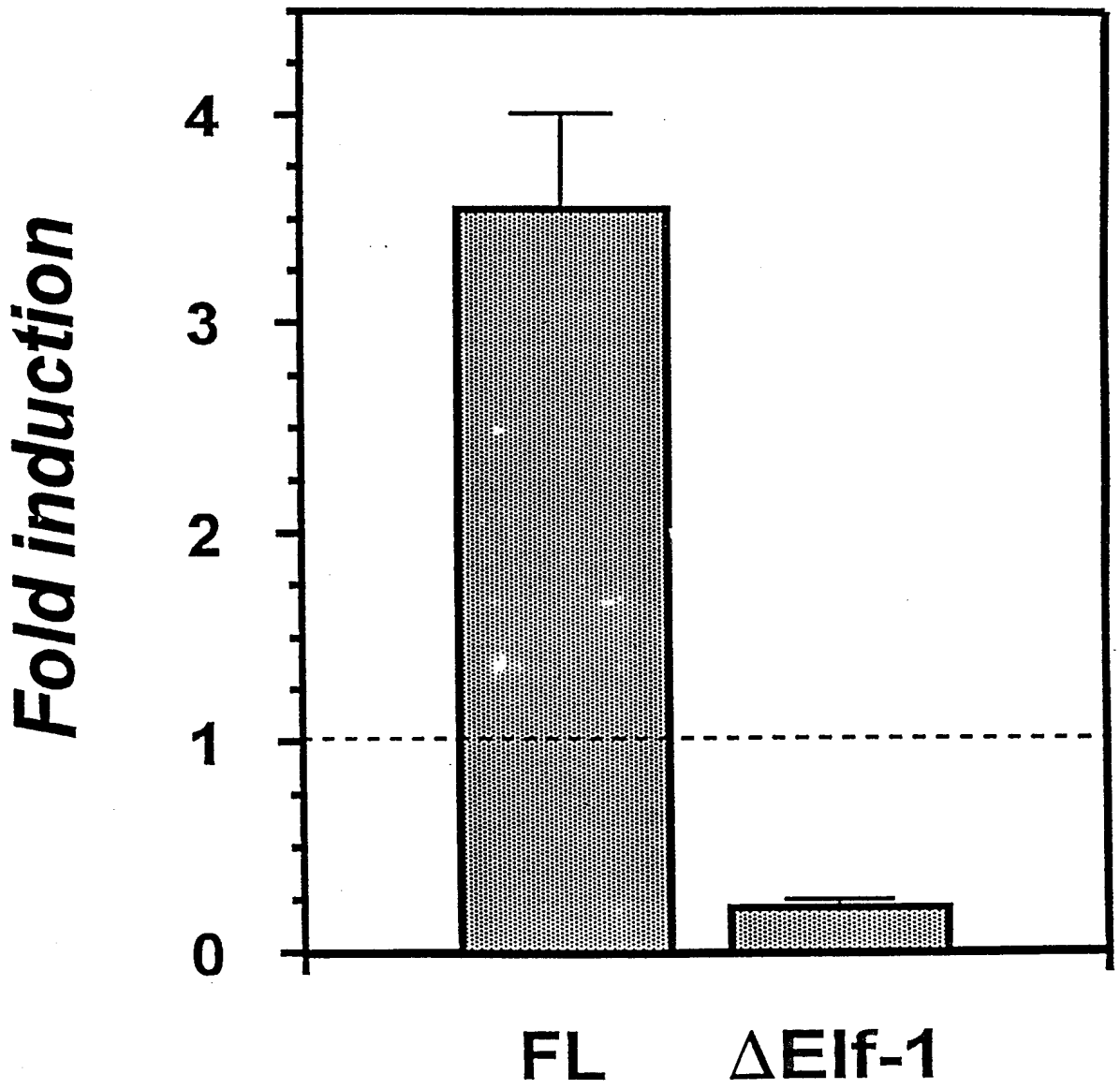
— Fig. 2A


Dimer
Net acidic
SPXX
Elf-1
DNA-binding
Δ1
Δ2
Δ3
Δ4
Δ5
Δ6
Δ7
Δ8
Δ9
ΔElf-1

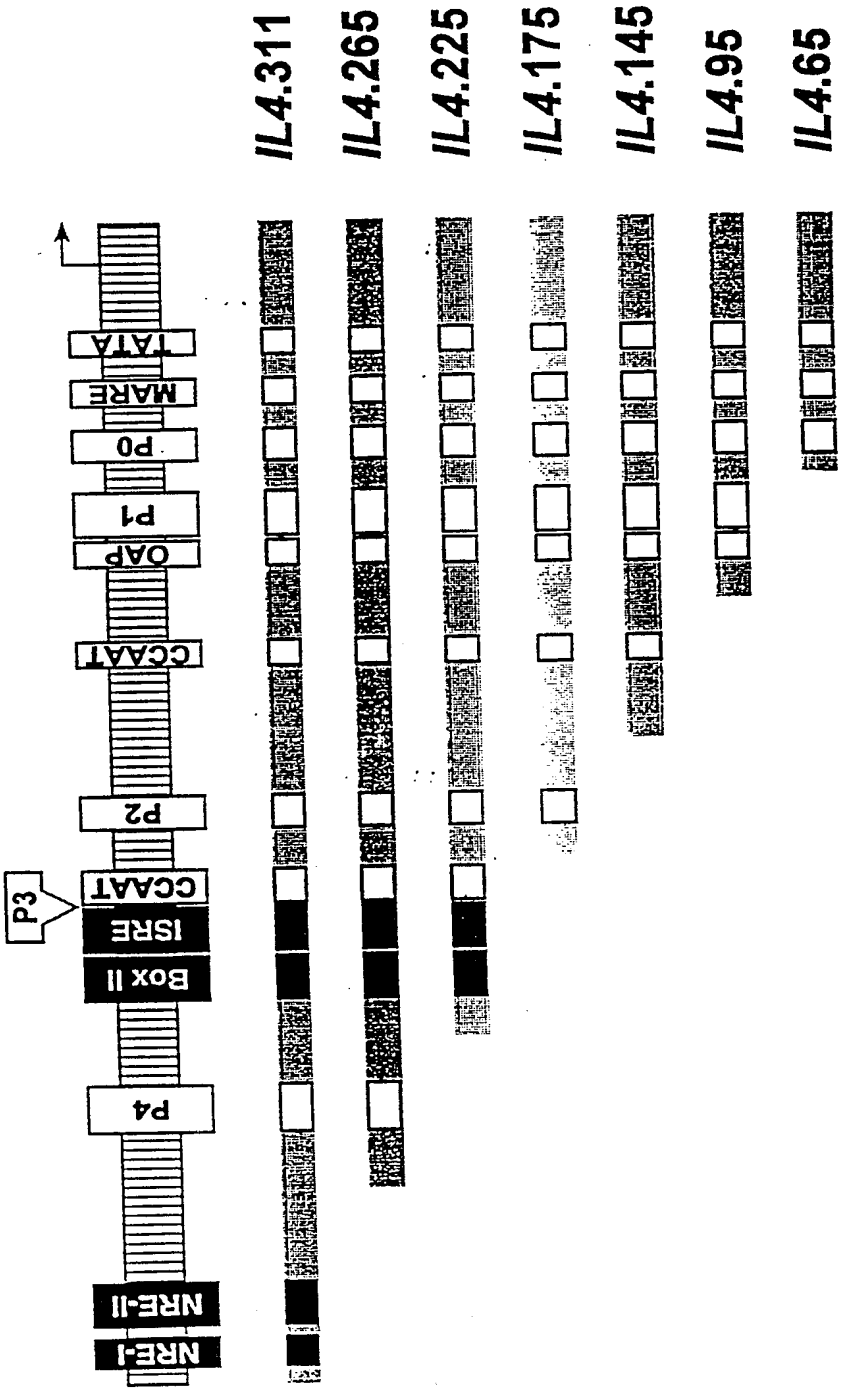
— Fig. 2B


Fold induction
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
FL Δ1 Δ2 Δ3 Δ4 Δ5 Δ6 Δ7 Δ8 Δ9
Cotransfected plasmid

— Fig. 2C



— Fig. 3A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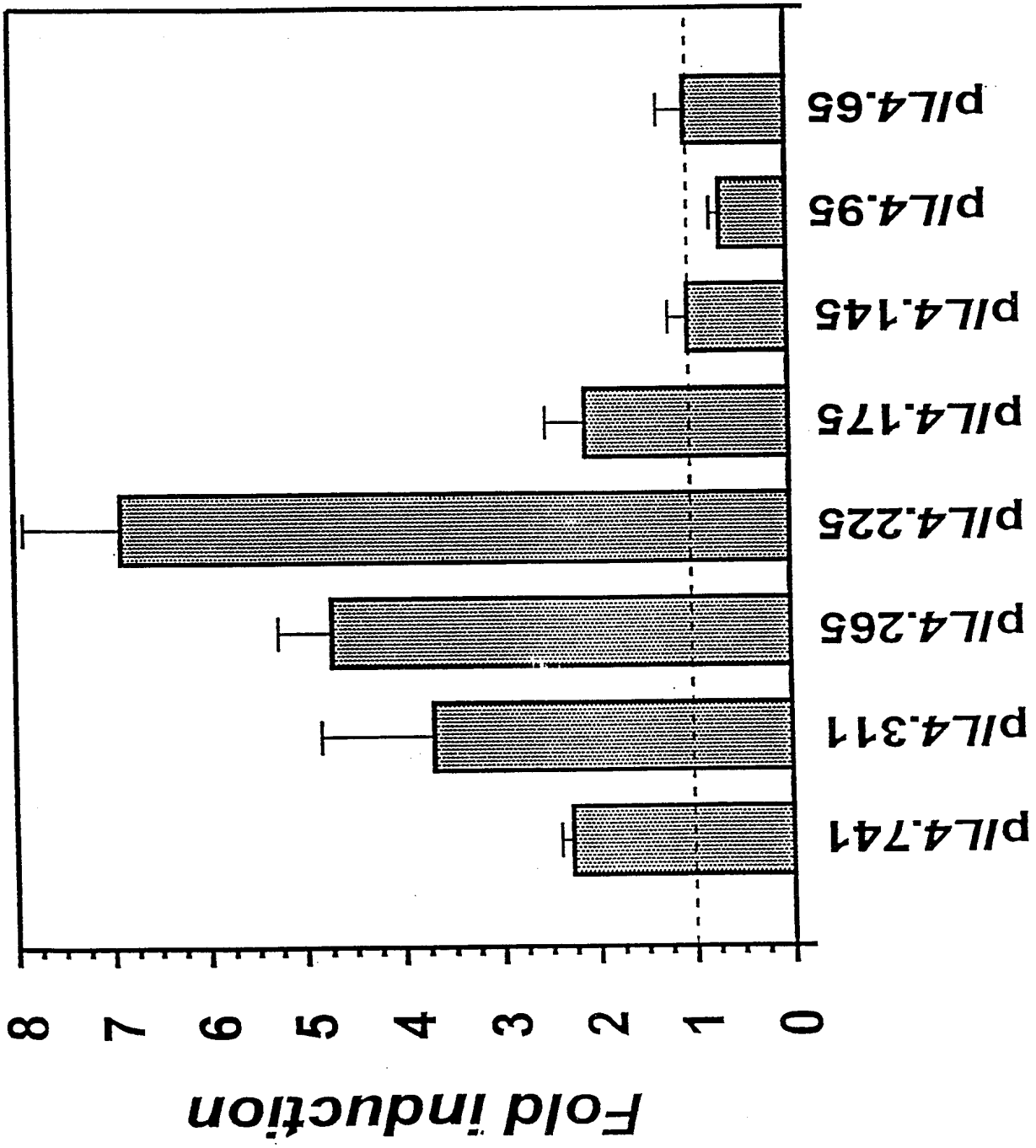
IL4.225
IL4.175
IL4.145
IL4.95
IL4.65
TATA
MARE
P0
P1
OAP
CCAAT
P2
CCAAT
P3
ISRE
Box II
P4
NRE-II
NRE-I

— Fig. 3B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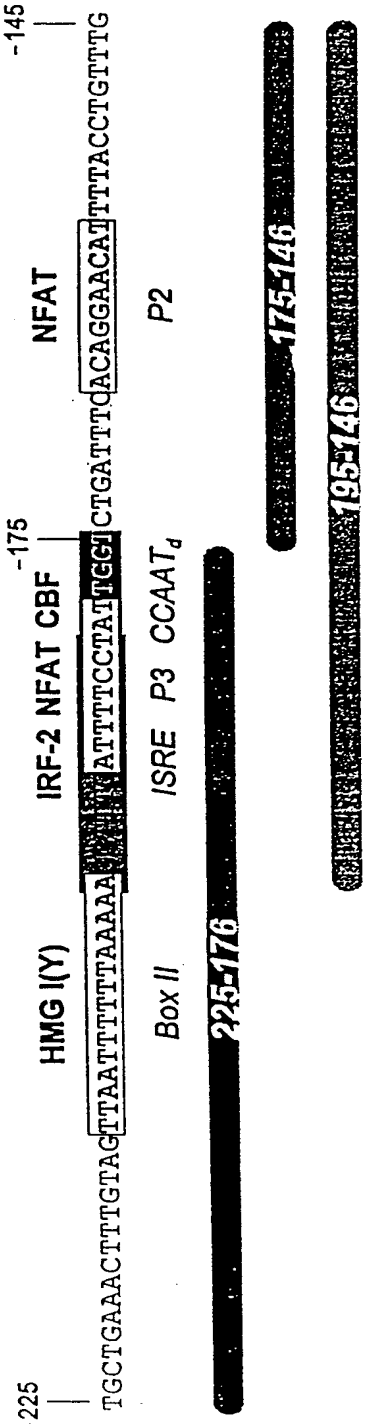
2
3
4
5
6
7
8
Fold induction

— Fig. 4A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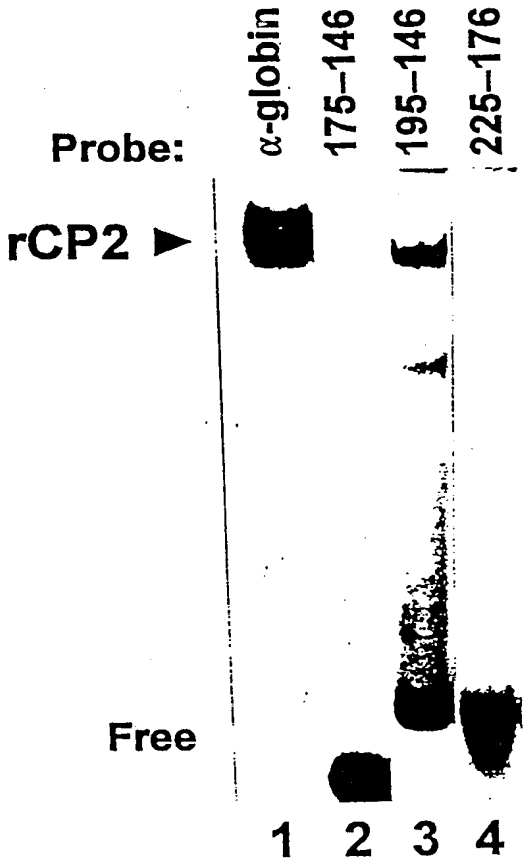
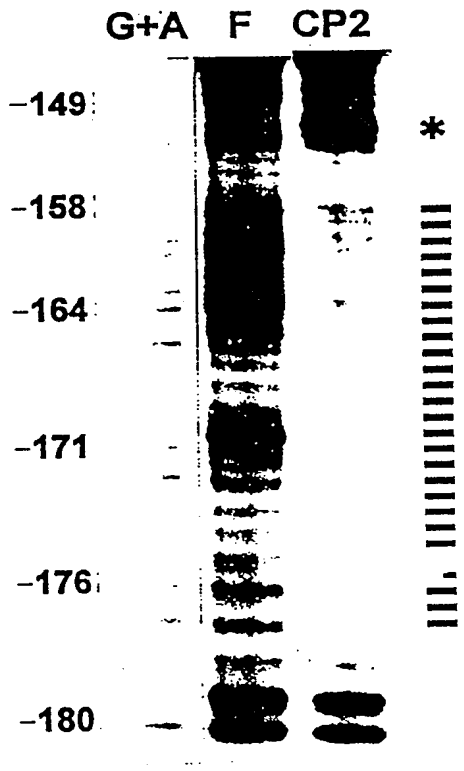
HMG I(Y)
IRF-2 NFAT CBF
NFAT
Box II
ISRE
P3
CCAAT$_d$
P2
225-176
175-146
195-146

— Fig. 4B



— Fig. 4C



— Fig. 4D

α-globin $^{-128}$AA**CAAG**TTTTTA-**CTGG**GT $^{-111}$

$^{-109}$AG**CAAG**CACAAA**CCAG**CC $^{-92}$

γ-fibrinogen $^{-68}$GA**CCAG**TTCCAG**CCAC**TC $^{-85}$

HIV $^{-4}$TA**CTGG**GTCTCT**CTGG**TT $^{+13}$

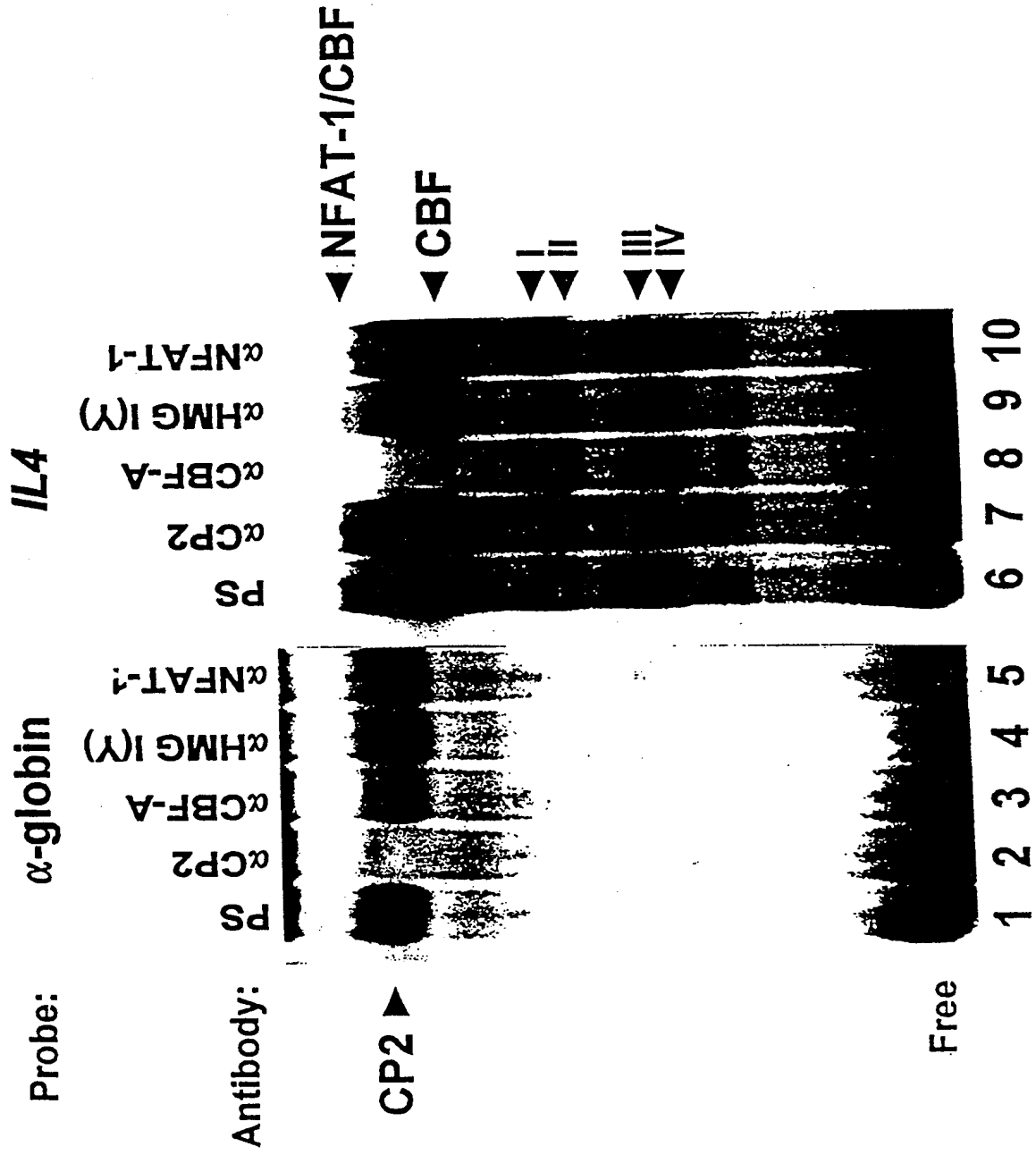
Ea $^{-3}$TT**CTGC**CTCAGT**CTGC**GA $^{+14}$

Cyp 2d-9 $^{-109}$TC**CTCC**CTATTC**CGGG**CC $^{-92}$

IL4 $^{-176}$GT**CTGA**TTTCA-**CAGG**AA $^{-160}$

Consensus **CNRGNNNNNNCNRG**

— Fig. 5A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I
II
III
IV
IL4
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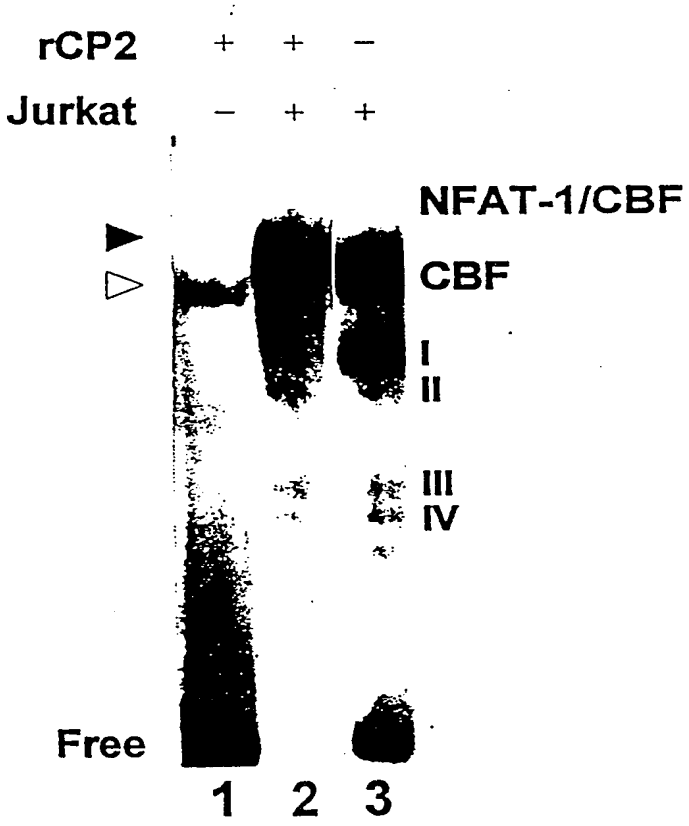
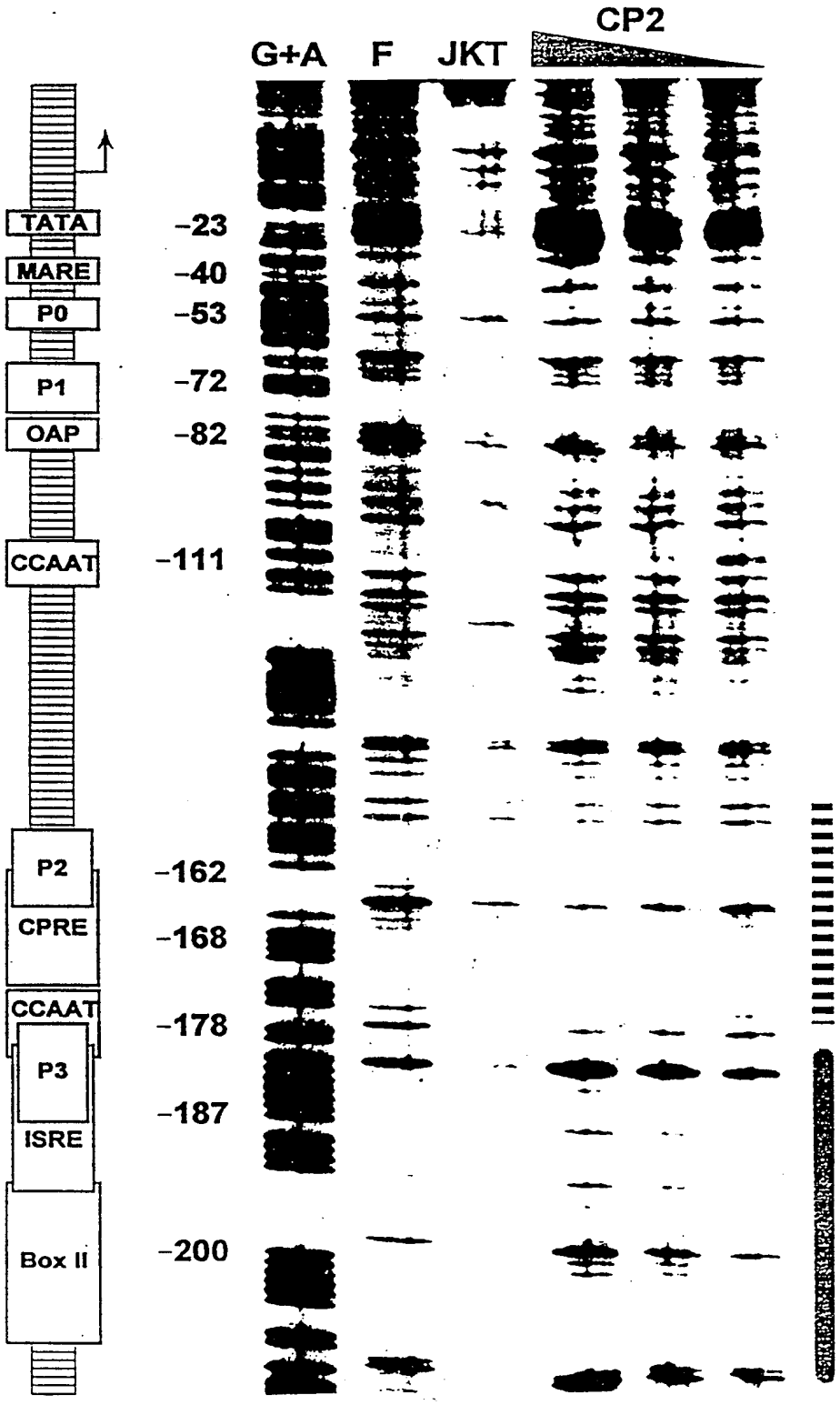
αHMG I(Y)
αCBF-A
αCP2
PS
6 7 8 9 10
α-globin
αNFAT-1
αHMG I(Y)
αCBF-A
αCP2
PS
1 2 3 4 5
Probe:
Antibody:
CP2
Free

— Fig. 5B



— Fig. 6A


G+A
F
JKT
CP2
TATA
MARE
P0
P1
OAP
CCAAT
P2
CPRE
CCAAT
P3
ISRE
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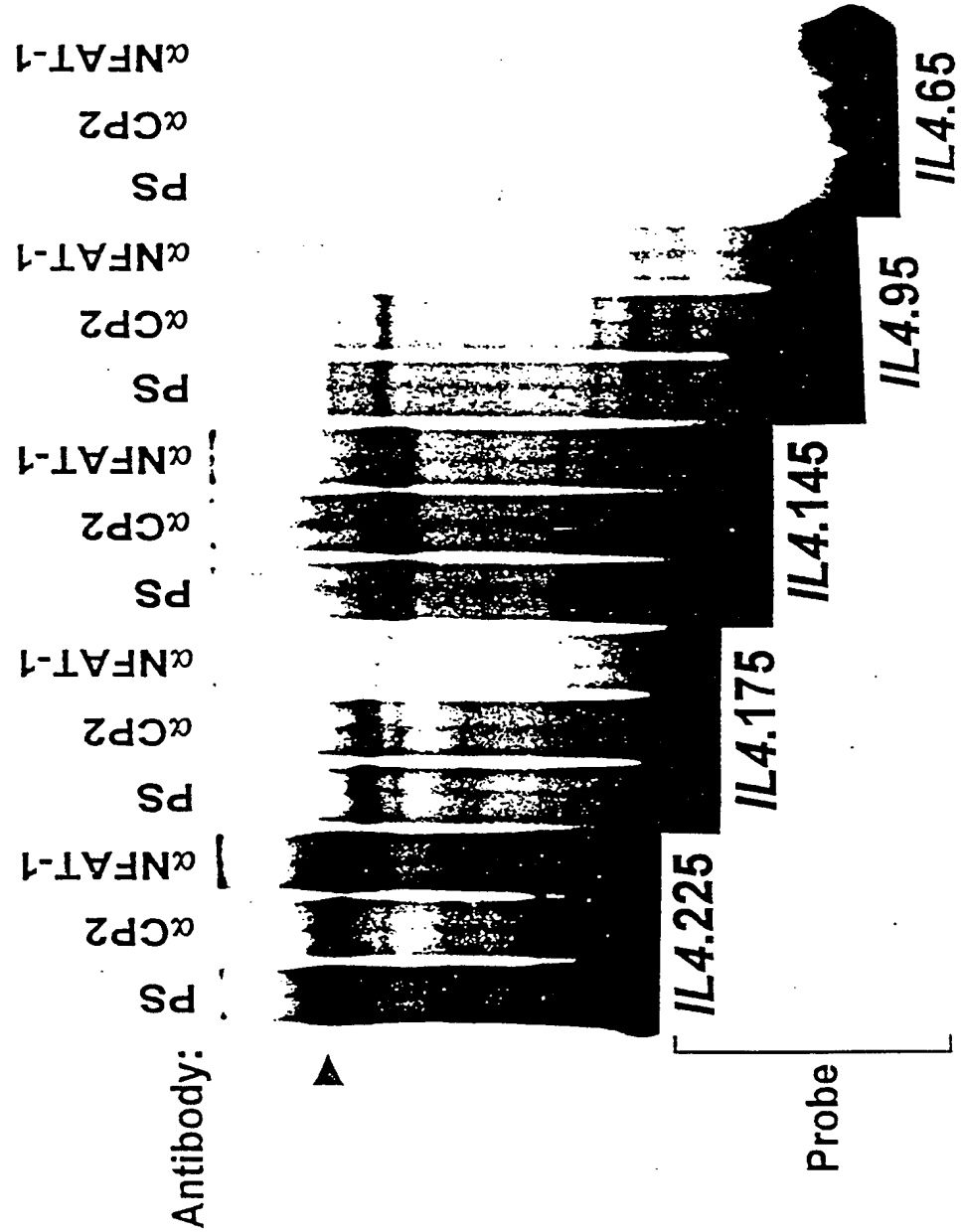
-40
-53
-72
-82
-111
-162
-168
-178
-187
-200

— Fig. 6B


Antibody:
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αNFAT-1
PS
αCP2
αNFAT-1
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αNFAT-1
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PS
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αNFAT-1
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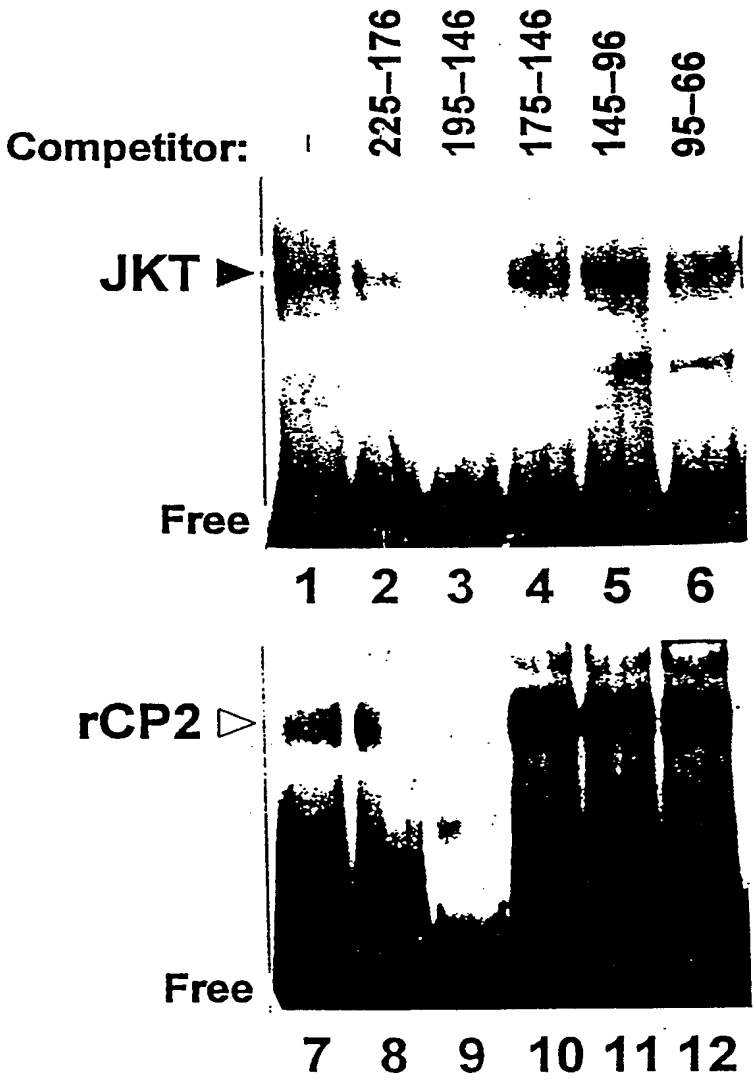
IL4.175
IL4.145
IL4.95
IL4.65
Probe

— Fig. 6C


Competitor:
-
225–176
195–146
175–146
145–96
95–66
JKT
Free
1 2 3 4 5 6
rCP2
Free
7 8 9 10 11 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03049

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A01N 43/04, 61/00; C12N 5/10, 15/00; C12Q 1/68
US CL :435/6, 172.3, 375; 514/2, 44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 172.3, 375; 514/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, SCISEARCH, CAPLUS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHAO, X., et al. Hepatic Nuclear Proteins that Bind cis-regulatory Elements in the Proximal Promoter of the Rat Corticosteriod-binding Globulin Gene. Molecular and Cellular Endocronology. 1997. Vol. 126. pages 203-212, see ntire document.	1-15
A	BRANCH A., A Good Antisense Molecule is Hard to Find. TIBS February 1998. Vol. 23, pages 45-50, see entire document.	1-15
A	STULL R., Antisense, Ribozyme and Aptamer Nucleic Acid Drugs: Progress and Prospects. 1995. Vol. 12. No. 4. pages 465-483, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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